Factors Predicting Response to Treatment in Chronic HCV Genotype 3 Patients

BY

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Dedication

This thesis is dedicated to my beautiful daughter Zahra - my strength, my inspiration.

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This thesis would not have been possible without the help and support of my Supervisor Professor Graham R Foster. His wisdom, knowledge and commitment to achieve the highest standard has inspired and motivated me. It is to him I owe my deepest gratitude.

I'm grateful to Dr. Jenny Waters who took out time to assist me despite his significant work commitments.

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Abstract

Studies to date have failed to identify the most effective treatment regimes for patients with chronic genotype 3 HCV infection. There is controversy regarding the role of cirrhosis in modifying response and disagreement regarding the impact of ethnicity on treatment outcome. Given the importance of genotype 3 HCV in the global epidemic and the lack of high quality research into this genotype, the purpose of this work has been to address some of the deficiencies in our understanding of the optimal management of this strain of hepatitis C. Specifically we have examined the following hypotheses:-

1) Patients from the Indian sub-continent (South Asians) will respond differently to therapy with pegylated interferon and ribavirin when compared to Caucasians.

2) An analysis of viral and host factors underlying differences between treatment sensitive and treatment refractory cohorts will reveal new insights into the virology of Genotype 3 HCV infection.

3) Increasing the duration of therapy in 'difficult to manage' patients with Genotype 3 HCV will improve response rates.

4) Whether non-invasive methods of identifying liver fibrosis are valuable in identifying the stages of fibrosis in Genotype 3 HCV patients.

Three different research methodologies were used to address these questions including a meta-analysis of factors associated with treatment failure in patients with genotype 3 HCV, virological and immunological studies on patients with genotype 3 HCV who had failed to respond to therapy and a clinical trial evaluating extended duration therapy in patients with Genotype 3 HCV infection and cirrhosis.

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List of commonly used abbreviations

ALT	Alanine aminotransferase
APRI	Aspartate aminotransferase (AST)-to-platelet ratio index (APRI)
CHB	Chronic hepatitis B
CHC	Chronic hepatitis C
CT scan	Computerised tomography scan
eCRF	Electronic case report form
ELF	Enhanced liver fibrosis test
EVR	Early Virological Response
EOTVR	End of treatment virological response
ELON	East London
FBC	Full blood count
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV IgG	Hepatitis C immunoglobulin G
HCV RNA	Hepatitis C Ribonucleic acid
HCV	Hepatitis C virus
IVDU	Intravenous drug users
MRI	Magnetic resonance imaging
PCR	Polymerase chain reaction
PegIFNalpha 2a	Pegylated Interferon alpha 2a
RT-PCR	Reverse transcriptase polymerase chain reaction
RVR	Rapid Virological Response
SAE	Serious adverse event
SVR	Sustained virological response
South Asian	Individuals originating from the Indian Subcontinent.
Treg	T regulatory cells

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Chapter 1

Introduction

1.1 Introduction

An infectious form of hepatitis that was not hepatitis A or hepatitis B was recognised over 30 years ago and was called non-A non-B hepatitis. Early studies showed that people presenting with episodes of acute hepatitis following transfusion of blood products did not have any serologic evidence of hepatitis A or hepatitis B and it was not until tests for hepatitis C virus (HCV) antibody became available in 1989 that it was recognized that the non-A, non-B hepatitis was caused by HCV. In 1989 the hepatitis C virus was identified by antibody screening of a phage library. Subsequent work showed that most patients with non-A non-B hepatitis were suffering from HCV [3].

The World Health Organisation (WHO) [4] estimates that 170-180 million people are chronically infected with hepatitis C, (about 3% of the total world population) and at risk of developing liver cirrhosis and liver cancer. An estimated 30% of chronically infected patients develop complications of cirrhosis or liver cancer (HCC) after 30 years. Thus chronic HCV infection poses a global health problem and, at present there is no vaccine that can prevent infection. However effective therapies are available and may prevent the complications of disease as well as reduce onward transmission by reducing the overall pool of infected patients [5].

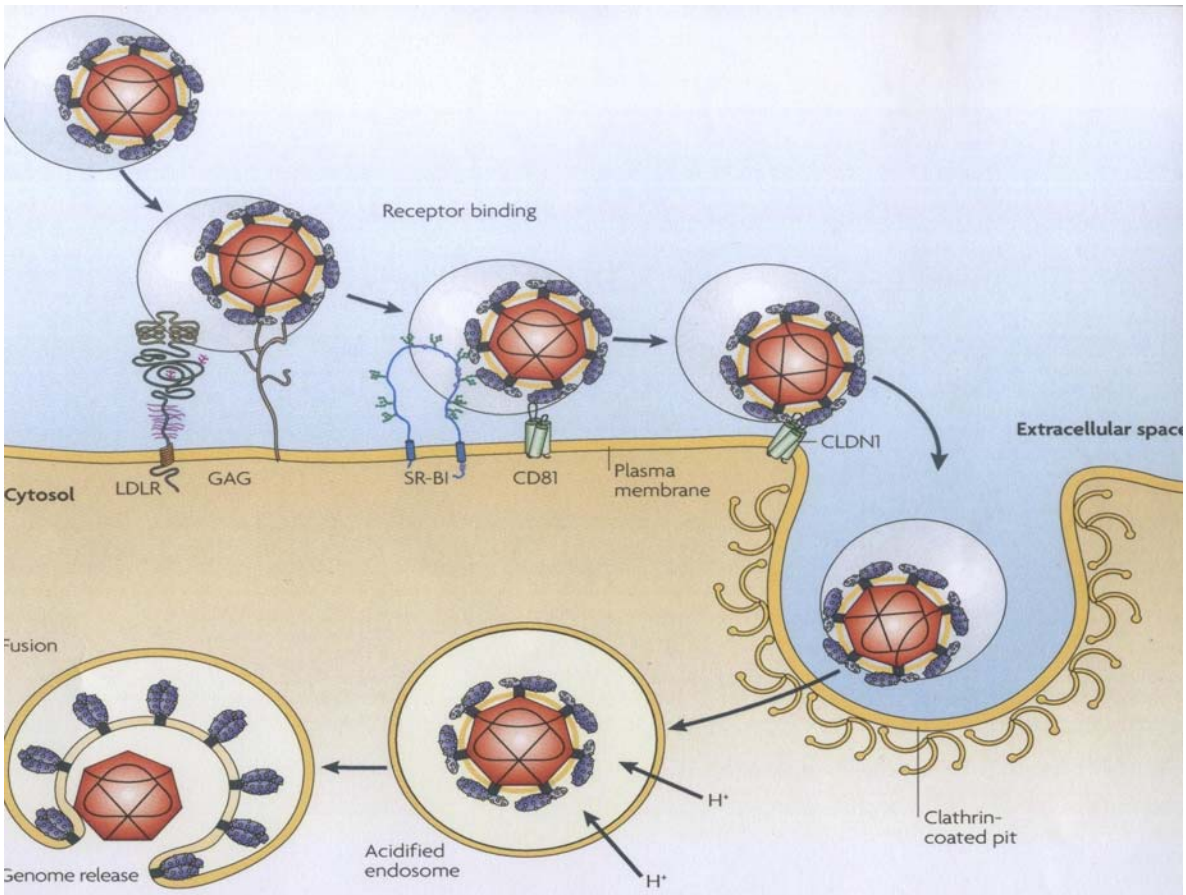
1.2 The Virus

Hepatitis C is an RNA virus of the Flaviviridae family and is most closely related to yellow fever virus and dengue virus. Hepatitis C virus replicates within the hepatocyte cytoplasm and possibly within B lymphocytes and spreads from cell to cell but is not directly cytotoxic [6]. In the infected liver approximately 10^{10}- 10^{12} viruses per day are produced [6]. The HCV envelope is derived from host membranes, into which the viral glycoproteins are inserted, and the nucleocapsid contains a positive-stranded RNA genome of 9600 nucleotides. The single stranded positive sense RNA genome of HCV is attached at both ends by conserved highly structured non translated regions (NTRs) and encodes a polyprotein precursor of 3000 amino acids. This is co-translationally and post-translationally cleaved into 10 structural and nonstructural proteins. The N-terminal region yields the structural protein core (C) and envelope proteins 1 and 2 (E1 and E2) which are required for the formation of the infectious viral particles [7]. The HCV envelope protein E2 binds to CD81 with high affinity and is thought to be one of the routes of entry of

HCV into cells. An experiment done by Wakita et al; 2005 showed that when Huh-7 cells were incubated with the infectious HCV virus, JFH-1 in the presence of CD81 specific or non specific antibodies, 48 hrs later CD81 specific antibodies reduced both the number of infected cells and the amount of HCV RNA confirming the important role of CD81 in HCV entry[8-10].

The non structural proteins NS2, NS3, NS4A, NS4B, NS5A and NS5B coordinate the intracellular processes of the virus life cycle. NS2 and the amino terminal domain of NS3 together form the protease that self cleaves the polyprotein between NS2 and NS3. NS3 has an amino-terminal serine-protease domain required for processing the polypeptide from NS3 to NS5 and at the carboxy-terminal of NS3 is a helicase/nucleoside triphosphatase domain. NS4A is the NS3 protease co-factor and NS4B contributes to the formation of the membrane associated replication complex. NS5A is a phosphoprotein that plays a crucial role in RNA replication and assembly and NS5B is an RNA dependent RNA polymerase (RdRp).The replicase complex which consists of NS3 to NS5B and cellular proteins copies the viral strand and serves as a template for excess RNA genomes. The absence of 3'-5' exonuclease proofreading activity of NS5B means that HCV RNA replication is error prone causing a high degree of genetic variability [7]. Inhibition of NS3 and NS5b function are key targets for the development of new antiviral agents.

Figure 1.1 summarises the replication and entrance of HCV virus[11].



Current model for hepatitis C virus (HCV) entry. Circulating HCV particles can be associated with low- and very-low-density lipoproteins (LP). Virus binding to the cell surface and entry may involve the low density lipoprotein receptor (LDLR), glycosaminoglycans (GAG), scavenger receptor class B type I (SR-BI), the tetraspanin protein CD81 and claudin-1 (CLDN1). CLDN1 functions at a late stage of cell entry, possibly at tight junctions of polarized hepatocytes. Internalization depends on clathrin-mediated endocytosis. Acidification of the endosome induces HCV glycoprotein membrane fusion. Little is known about the uncoating process, which results in genome release into the cytosol.

Six major HCV genotypes and 100 subtypes have been identified throughout the world on the basis of molecular relatedness of conserved and non-conserved regions. Until recently it has not been possible to infect cells with HCV so an understanding of HCV replication has been derived from the use of a replicon system in which either the non-structural or the full-length genome of HCV was transfected into the human hepatoma cell line Huh-7, resulting in few colonies of cells containing replicating HCV RNA. HCV RNA replication has also been achieved in HeLa, 293T, HepG2 and mouse hepatoma cell lines, although only low level replication was seen [12]. Chimpanzees are the only species other than man that can be infected with HCV and thus have proved important in the study of natural history of HCV [8].

1.3 HCV genotypes and subtypes

Early work has shown that HCV has substantial nucleotide sequence diversity distributed throughout the viral genome. The phylogenetic analysis of the nucleotide sequences derived from part of the gene encoding a non-structural protein (NS-5) has provided evidence for six major genotypes of HCV. They were derived by using samples from chronic HCV infected patients and from blood donors. Comparison of published sequences of HCV has led to the identification of a number of distinct virus types and subtypes, that may differ from each other by as much as 33 % over the whole viral genome [13]. The HCV genotype is of clinical significance as it helps to tailor the treatment regimen and helps in monitoring response to treatment [5, 14]. HCV is classified into six major genotypes with more than 50 known subtypes. Genotype 1 is the most common genotype present globally being predominantly found in America, Japan, Korea, Australia & New Zealand whilst genotype 3 is highly prevalent in the Indian subcontinent[14]. Genotype 4 is predominantly found in the Middle-East and Egypt

Genotype 5 is commonly found in South Africa while genotype 6 is found in West Asia [5]. The HCV genotype 1 has been strongly associated with disease progression and HCC [5]. Recent studies have shown a strong association between genotype 3a and HCC in patients from Pakistan where genotype 3/3a is found to be the predominant genotype [15-17]. None of the large scale studies have shown any association between HCV genotype and disease progression[18] and it is probable that all genotypes contribute equally to adverse outcomes, although formal studies have not been completed for all genotypes. The studies which have shown a strong association with cancer, genotype 1b and genotype 3 come from an area of the specific genotype endemicity and should not be used to classify only these genotypes as a risk factor associated with the progression of disease [19].

1.4 HCV Quasispecies

In HCV infected individuals, the virus circulates as a population of many different but closely related viral variants which are referred to as ‘‘quasispecies’’. New variants are continuously generated during viral replication, as a result of errors made by the viral RNA-dependent RNA polymerase that lacks proofreading activity [20]. Studies of HCV quasispecies and their relation

with the outcome of antiviral therapy have been based mostly on the interferon sensitivity determining region (ISDR) [21].

In a study of 153 genotype 1 patients, quasispecies analysis of the HVR1 has shown that EVR is associated with elevated HCV quasispecies (QS) diversity and complexity. However the limitation of the study was that it only followed patients through the first 12 weeks of therapy and there was an inability to correlate HCV QS heterogeneity with SVR [22]. Also by the use of Ultra-deep sequencing technique in 27 HCV treatment naïve Japanese patients genotype 1 patients, it was found that Immediate responders showed a significant decrease of genetic complexity spanning all regions of the genome while nonresponders showed no significant change in the genetic complexity in any of the HCV genomic regions[23]. The hypervariable region 1(HVR1) located in the amino-terminus of the HCV envelope glycoprotein E2 is the most variable part of the HCV genome. Studies suggest that the HVR1 is susceptible to immune pressure involving neutralising antibodies and allowing the selection of escape mutants [24] which may contribute to viral diversity, although the contribution of these mutations to disease response is unclear.

1.5 Immune responses that control viral replication

The mechanisms that regulate the induction of fully integrated innate and adaptive immune responses have to be perfectly timed from the early stages of HCV infection to control, and finally to clear, the virus. Since this seems to be the exception rather than the rule, as more than 80% of infections lead to viral persistence, it is thought that efficient strategies to interfere with innate and adaptive immune responses must have been developed by HCV. Induction of peripheral tolerance, exhaustion of the T-cell response, infection of immunologically privileged sites, inhibition of antigen presentation, interference with the interferon (IFN) system, down-regulation of viral gene expression, viral mutations that abrogate, anergise or antagonise antigen recognition by virus-specific T cells are some of the possible mechanisms that have been postulated to explain the high rate of HCV persistence. An additional explanation could be based on the fast kinetics of infection in relation to the stimulation of the adaptive immune response during the early phase of infection [8, 25].

1.6 The innate immune response in HCV infection

In the early stages after being infected with HCV, type I interferon is produced by the infected cells and induces a rapid interferon response creating an antiviral state in the liver and

surrounding cells. It is not known how HCV survives in spite of such a strong initial response by the infected cells. In the chimpanzee model within seven days of infection with HCV there is an increase in expression of the interferon stimulated genes (ISG) which include 2-5 oligoadenylate synthetases (2-,5 OAS) 1 and 2, IFN stimulated gene 56 (ISG56, p56), MxA, and 6-16 [26]. Many of the same genes are induced in cell lines bearing HCV replicons following treatment with type I IFNs [27]. Type I IFNs signal through the IFN-α receptor to activate the Jak kinases Tyk2 and Jak1; this leads to phosphorylation of STAT1 and STAT2, which associate with IRF-9 to generate the transcription factor ISGF-3 which then translocates to the nucleus and ISGF-3 stimulates the transcription of more than 300 IFN-stimulated genes (ISGs), some of whose products play direct antiviral roles. The interferon stimulated genes (ISGs) with direct antiviral activity include 2-5 OAS, RNAse L, protein kinase R (PKR) and ISG56 [8] .

Cells of the innate immune system include natural killer cells (NK), NKT cells and myeloid cells which are also present in the liver. NK cells are activated early in viral infections and are known to produce IFN-alpha which directly inhibits viral replication, and attract lymphoid and inflammatory cells to the site of infection. IFN-alpha and TNF alpha also lead to activation of myeloid dendritic cells and defects in this mechanism have been reported in HCV infected patients [28]. Recent studies have shown that their levels or activation state may be altered in the liver or in the peripheral blood during persistent infection [8, 29].

1.7 The adaptive immune response in HCV infection

Cellular immunity to HCV has been shown to play an important role in the control and eradication of infection. The induction and maintenance of effective antiviral immunity requires functional antigen specific CD4+ and CD8+ T cells. The CD8+ T cells have both cytolytic and non-cytolytic anti-viral effector functions, the latter of which is mediated by production of cytokines such as IFN-gamma and TNF-alpha. In both human and chimpanzee studies, HCV specific CD8+ T cell responses in acute spontaneously resolving HCV infection are vigorous and target multiple epitopes, whereas HCV specific CD8+ responses in those individuals who evolve to chronic infection are lower in frequency and target only a few epitopes [8, 30]. There is evidence that without sustained CD4+ T cell help, CD8+ T cells cannot keep pace with viral replication and ultimately viral escape mutations develop [10, 30, 31] .Wertheimer et al (2003) reported that patients who had spontaneously eradicated the virus showed persistent $CD4^+$ and

$CD8^+$ T cell responses to specific peptides [8, 32] ,suggesting that a broad, multispecific immune response is associated with control of viral replication.

The patterns of CD4+ and CD8+ T cells in patients with resolved HCV infection contrast with the very limited number of HCV epitopes targeted by T cells from patients with chronic infection. In those who are chronically infected, the number of epitopes recognised decreases [10]. In patients with chronic HCV infection T cell responses seen during acute infection may be lost and there is a decrease in the number and function of responding T cells in peripheral blood [8, 33]. Specific loss of CD4+ T cell responses predicts recurrence of viraemia and establishment of chronic infection [34]. Whilst in established chronic HCV infection, CD8+ T cells which are specific for HCV epitopes are abundant and make up about 1%–2% of total CD8+ T cells in the peripheral blood or liver [8, 34, 35] .

Most of these CD8+ T cells are deficient functionally in that they have a limited ability to produce IFN-gamma, to proliferate, or to kill cells, the so-called 'stunned' T cells [8].

Activated HCV specific cytotoxic T lymphocytes CTL accumulate in the liver of patients with chronic hepatitis C and play a major role in the inhibition of viral replication. A recent study showed that a dynamic CTL response specific to the epitope HCV NS3 1073-1081 could be detected in patients with severe hepatitis but not mild hepatitis suggesting that CTL responses may be responsible for liver damage [32, 36].

1.8 How viruses avoid immune responses

Following exposure to HCV, HCV RNA genomes appear in the plasma within a few days of infection and typically peak 6–10 weeks later regardless of outcome. A pattern of poorly controlled viraemia predicts persistence that may be explained, at least in part, by the failure of some individuals to generate detectable CD4+ and CD8+ T cell responses [37]. HCV can outpace the adaptive immune response, behaving differently from other viral infections such as HIV, CMV, where T cell responses can be detected earlier after infection. The delay in the adaptive immune response needs to be studied in detail as there are limited animal and human studies defining a clear HCV specific T cell response [8].

Studies have shown that patients with chronic HCV infection have elevated circulating frequencies of regulatory T cells (Tregs) that may impair HCV specific responses but to date no study has examined their role during antiviral therapy[8, 31].

It takes a long time to develop a virus specific neutralising antibody in HCV infection and amino acid mutations within the hyper variable region (HVR1) of the HCV E2 region are highly diverse and may contribute to the persistence of infection by facilitating escape from the neutralising antibodies [38].

It has also been suggested that mutations in epitopes of HLA class I or II restricted T cells contribute to viral persistence by interfering with the recognition of HCV infected cells. A mutation of an amino acid within the epitope of the NS3 region which is recognized by Th1 cells resulted in a shift in cytokine secretion patterns from Th1 to Th2 cells and Th2 responses lead to decreased antiviral responses. CD8 T cells could recognise parental epitopes but not variant sequences .The variant sequences could not induce a CD8 response efficiently in vitro [8, 10].

T cell responses can be detected in the acute phase and chronic phase of the infection. CD8+ cells infiltrate the liver and are unable to eradicate the virus. CD3+ which is very important for T cell functioning has been reported to be reduced on the surface of peripheral lymphocytes. As a result, the T cells are defective and there is impaired production of interferon gamma, low perforin content and decreased proliferation and cytotoxicity [27].

NK cells functions are controlled by inhibitory receptors for major histocompatability complex (MHC) class I, including the killer cell immunoglobulin-like receptors (KIR). KIR2DL3 in combination with its cognate human leukocyte antigen (HLA)-C ligand has been shown to be associated with spontaneous resolution of viraemia following chronic HCV infection [39, 40].

Dendritic cells play an important role as antigen presenting cells (APCs) and link innate and adaptive immune responses. The two major subsets of dendritic cells are myeloid DCs (mDCs, DC1), which predominantly secrete IL-12 and TNF-alpha, driving naive CD4 cells to a Th1 phenotype, and plasmacytoid DCs (pDCs, DC2). They secrete large amounts of IFN-alpha in viral infections and drive naive CD4 T cells toward a Th2 phenotype. In a study where dendritic cells were taken from uninfected individuals and then stimulated with HCV core and E1 proteins it was seen that they were impaired in their capacity to stimulate T cell responses [34, 41], suggesting that these proteins may modify the activity of dendritic cells. Various studies on DC function and the outcome from chronic HCV infection have produced contradictory results. Some studies have examined individuals with a history of spontaneous clearance of HCV infection and individuals with chronic HCV infection, and compared these to each other or to non-infected controls. These studies have shown that myeloid dendritic cells from the peripheral

blood of chronically HCV infected patients have an impaired capacity to stimulate allogenic T cell responses and secrete IL12[30] [42], perhaps suggesting that dendritic cell dysfunction is involved, at least in part, with the immune defects associated with chronic HCV infection.

HCV specific CD8+ T cells can survive for years in the liver and may contribute to progressive liver disease. With regards to viral replication, it is noteworthy that the level of viraemia remains relatively stable over time in subjects with chronic infections [30]. In patients with established chronic HCV infection, the frequency of antigen specific T-cells in the peripheral blood is dramatically decreased, T-cell's functional impairment could be the result of the combined effect of different mechanisms, including prolonged exposure of T cells to viral antigens, immunosuppression by viral gene products and impaired DC accessory function.

Another explanation for T cell exhaustion might be the deletion of virus specific T cells in the presence of continuous high viral load as has been demonstrated for lymphocytic choriomeningitis virus (LCMV) infection in adult mice (Moskophidis et al., 1993). In this model, T cell depletion was often preceded by unresponsiveness of T cells, e.g., their inability to produce IFN upon antigen stimulation as well as T cell receptor (TCR) down-regulation (Gallimore et al., 1998; Ou et al., 2001; Zajac et al., 1998).It remains unclear whether this mechanism plays a role in HCV infection. Primary T cell failure and T cell exhaustion may both contribute to viral persistence [8].

Summary: Clearance of HCV

Clearance of HCV either spontaneously or following interferon therapy is associated with a broad, potent immune response. In contrast chronic infection is associated with a muted Th1 response. The mechanisms underlying this immunological failure are not yet clear but many different defects in the response to HCV have been described. It is not clear which is the dominant mechanism and whether they all play an equal role.

1.9 Epidemiology of HCV

1.9.1 Global epidemiology

High quality studies in the developed world have characterised the infected population in great detail but in the developing world much less is known about the burden of disease and the people who are infected. Studies in the developed world have shown that HCV is chiefly a disease of drug users. The National Health and Nutrition Examination Survey which was done in the United States (1999-2002) has shown an overall prevalence of anti-HCV of 1.6% of the 15079

participants tested. IVDU was found to be the strongest risk factor associated with HCV. The prevalence was increased in non hispanic black (3%) and risk factors for infection include intravenous drug use (57.5%) and a history of blood transfusions before 1992. Prevalence rates were higher in men (2.1%) than women (1.1%) and there was a clear age effect with the prevalence in young patients of 20-29 years of age being 1.0%. compared to 4.3% in people between the age of 40-49 years [43]. This study did not look at homeless people and people who were in jail in the United States and therefore the figures cannot be used to estimate the overall prevalence of HCV in the country.

Data from Egypt has shown iatrogenic spread of HCV but little data is available from other developing countries. Egypt has reported the highest prevalence of HCV worldwide, ranging from 6% to more than 40% differing amongst different regions of the country. Jimenez et al have shown in a case control study of 100 cases and 678 controls that iatrogenic spread of HCV is liable for 34.6% of the new infections of HCV in Egypt [44, 45]. The mode of iatrogenic spread is believed to be contaminated medical equipment used to treat schistosomiasis and the Egyptian prevalence may therefore be unique.

A study done in 1997 participants in 17 villages in Pakistan has shown that 23.8% (n=476/1997) were anti-HCV positive. A very high prevalence of HCV was found in the study population and the risk factors that were identified were unsafe health care practices (dental procedures,blood transfusions and therapeutic injections), while specifically amongst men extramarital sex, shaving from a barber and hospital admissions were found to be associated with HCV infection [46].

In other studies in developing countries IVDU appears to be a significant risk factor for chronic HCV infection, although it is not clear whether this is related to ascertainment bias (i.e. health agencies believe IVDU to be a major risk factor and therefore focus questions and testing on those who may have injected drugs)**.** However, of approximately 13.2 million IVDU's worldwide, it has been estimated that 10 million injection drug users (78%) live in developing countries [47], hence other risk factors must play a role in the developing world**.** Xian Xia et al have shown that the prevalence of HCV infection among IVDUs in China was 61.4% (95% CI 55.7–67.2%)[48].

Data from other developed countries is often of poor quality and involves relatively small numbers of patients. However a five year retrospective blood donor study in India has shown of

the 94,716 donations, the prevalence of HBV seropositive donations was 1353 and that for HCV was 537, with the prevalence rates of 1.43% for hepatitis B surface antigen (HBsAg) and 0.57% for HCV[49]. Another study of voluntary blood donors in India has shown of 15,898 healthy voluntary blood donors 249 were found to be reactive for anti HCV antibody, showing an overall prevalence of 1.57% [50]. Table 1 shows the current estimates for the prevalence of HCV throughout the world based on a variety of studies collated by the WHO.

WHO Region	Total Population (Millions)	Hepatitis C prevalence Rate %	Infected Population (Millions)
Africa	602	5.3	31.9
America	785	1.7	13.1
Eastern Mediterranean	466	4.6	21.3
Europe	858	1.03	8.9
South-East Asia	1 500	2.15	32.3
Western Pacific	1 600	3.9	62.2
Total	5 811	3.1	169.7

Table 1.1: Hepatitis C estimated prevalence and number infected by WHO Region. Source: Weekly Epidemiological Record. N° 49, 10 December 1999, WHO[51]

1.9.2 HCV in the UK

In the UK there have been no wide spread prevalence studies. In general two populations have been studied – some studies focussed on IVDU and others have looked at the general population. A typical study examining the prevalence of HCV in 1949 IDU's in Glasgow, found a prevalence of salivary antibodies, indicating chronic HCV infection in 61% (95% confidence interval (CI) 59%–63%)[52] and other studies using similar approaches have also found very high prevalence rates. A cluster randomized controlled trial (RCT) was done in selected

specialist drug clinics and prisons in England and Wales to test the use of dried blood spots for diagnostic testing and showed that HCV positive antibodies were detected in 320 of the 1034 (32%) patients during the trial. A total of 2700 of 51 000 (5%) specimens were tested HCV positive in the 11 laboratories [53]. In a prospective cohort study which was done in 428 IDU it was found that the prevalence of antibody to hepatitis C virus was 43.7% (n=187/428) in the study population [54].

General population studies in the UK have been relatively few and have focused on specific groups,that may or may not be representative. A study looking at the prevalence of hepatitis C in 4729 pregnant women booking for antenatal care at a London hospital between 1997-1999 found an overall prevalence of HCV of 0.8% (n=38/4729) in pregnant women [55]. This was a small scale study where women were presenting themselves to the hospital and would be subject to selection bias. This group of pregnant women were considered a low risk group and thus the results may not represent the whole population. Another study looking at the prevalence of chronic HCV infection was done by testing stored sera for HCV from different time periods.The overall anti-HCV prevalence was found to be of 1.07% (39/3647) in 1986, 0.55% (31/5634) in 1991 and 0.70% (45/6401) in 1996. In 1996, prevalence was 11/865 (1.27%) in the Greater London area, which was higher than the prevalence of 34/5536 (0.61%) in the rest of England and Wales [56].

Given the ethnic diversity of the UK it is likely that the prevalence of HCV in immigrant communities will be greater than that seen in the indigenous population. A study which looked at the prevalence of HCV in people born in Pakistan of 2.7%(n=67/2471) and Bangladesh 0.44%(n=4/897) found an overall prevalence of 1.5% (n=78/5162)[57]. Thus the overall prevalence of HCV in the UK is not clear but an attempt was made to collate all the available data in 2010 and an estimate of the overall number of people who were HCV antibody positive was made at 191,000(95% CrI:124,000,311000) and approximately 142,000 were estimated to have chronic HCV infection (95% CrI:90000,231,000) in England and Wales although the confidence intervals were broad and the overall prevalence remains unclear with particular deficiencies in the available data relating to prevalence in ethnic minority groups [58-60].

Diagnostic rates for HCV in the UK are increasing. According to the UK Health Protection Agency report published in 2008, the number of reported laboratory confirmed cases of HCV infection was 7540 in 2007 as compared to 6721 cases in the previous year. This represents a

12% increase in detection rates in England [61]. A pilot study carried out in GP surgeries in the UK has shown that of the 234 volunteers tested, 35 were positive for hepatitis C antibodies, leading to a prevalence of 15% which was higher than the 4% suggested by tests carried out in GP surgeries in 2008[62]. Although this was a small study and there maybe an element of selection bias where GPs may be selectively testing those people who are at high risk i.e. 31/234 (13.2%) patients who tested positive for anti HCV were IVDU's, resulting in a higher prevalence than that found in the UK. Data from the Health Protection Agency 2009 has shown that 8196 people were diagnosed with hepatitis C infection and it is thought that as many as 100,000 people in England and Wales are not aware that they are infected[62].

1.9.3 HCV in immigrants in the UK

A recent large community based study performed in the UK on South Asian immigrants predominantly from Pakistan showed a prevalence of anti-HCV of 2.7% in people from Pakistan living in the UK with an increased risk of chronic HCV infection found in people born in the Punjab province of Pakistan[57]. More prospective community based studies are needed to assess the prevalence of HCV in ethnic minorities living in the UK.

1.9.4 Prevalence of HCV-Europe excluding the UK

The prevalence of chronic HCV infection has changed substantially during the last 15 years. The main factors that have caused these changes are: increased blood transfusion safety, improvement of healthcare conditions, increase of intravenous drug use and immigration to Europe from endemic areas[63]. In France a screening campaign in general practices screened 11,805 subjects of which 101 tested positive for HCV and showed prevalence rates of 1.3%[64]. However a study done in Southern Italy showed an increase in prevalence 12.6% (n=170/1,352 subjects) [65] perhaps indicating that the prevalence may be increased in some European Union countries. The high prevalence of HCV in Southern Italy has been attributed to the reuse of glass syringes, the use of unsterilised equipment during surgeries and may also be attributed to the increase in intravenous drug use.

In selected populations in Europe the prevalence of HCV may be very high. For example a study involving the screening of 2796 patients in 43 dialysis units in Germany showed a prevalence of 7%[66] whereas as a multicentre study of 984 patients undergoing dialysis from Southern France has shown a prevalence of HCV of 23.6%(n=232/984)[67]. It is likely that both the high

prevalence of blood transfusions in patients with renal failure as well as nosocomial transmission have played a role in these high prevalence rates.

1.10 Prevalence of HCV-Egypt

Egypt has an overall rate of 15%- 20% of HCV seropositivity, an epidemic which was caused by parenteral anti-schistosomal treatment which involved 12-16 intravenous injections of a tartar emetic [68]. A study found that of the 1023 patients referred for chronic liver disease, 752 (73.5%) had antibodies to HCV and the prevalence of HCV in patients with schistosomiasis or HBV infection was high. Studies have shown that Egyptian blood donors have the highest prevalence of HCV infection seen anywhere in the world i.e.11%-22%[69]. The correlation between Bilharzia and HCV was shown in a study of 8499 Egyptians where the degree of exposure was estimated from 1961–86 from the Egyptian Ministry of Health data. A significant association was found between seroprevalence of antibodies to HCV and the exposure index (1·31 [95% CI 1·08–1·59]; p=0·007) was seen across Egypt. The cohort specific HCV prevalence was lowest in children and young adults than in older cohorts showing a decrease in prevalence once the parenteral treatment was replaced by oral therapy. Lack of appropriate sterilisation procedures and reuse of therapeutic injections have led to the spread of HCV and re-infection with HCV in Egypt [68].

1.11 Prevalence of HCV-Africa

Few high quality studies have been performed in Africa. Previous studies had shown a prevalence of 6.5% in Central Africa but a study looking at 947 pregnant women in Gabon, central Africa, found that 20 women tested positive for HCV giving a prevalence of 2.1%[70]. The prevalence of HCV in Sub-Saharan Africa is 3.0%, West Africa has an estimated prevalence of 2.4%, and Southern and East Africa with the lowest estimated prevalence of 1.6% [71]**.**

1.12 Prevalence of HCV-Pakistan

Pakistan has an overall population of 170 million with an estimated 10 million people (6% of the total population) infected with HCV[72]. Zaheer Uddin et al first reported HCV in Pakistan in 1992 when the group tested 45 histologically confirmed cases of chronic liver disease for anti-HCV antibodies and found six patients to be anti-HCV positive. A study in the Agha Khan hospital in Pakistan found a high prevalence of anti-HCV (1.18%, 198/16,705) in healthy blood

donors [73, 74]. Looking at the prevalence of anti-HCV amongst young men between the ages of 17-22 across two military hospitals in Pakistan, 1.7% (95/5707) of men tested positive for anti-HCV [75].

In another study looking at the prevalence of anti-HCV in Pakistani women and materno-fetal transmission, antibodies to HCV were detected in 1.8% (108/5902) of pregnant women attending for booking visits and in 16% (89/548) of women attending the local hospital for elective gynaecological procedures. No vertical transmission was found in the babies that were born to anti-HCV positive mothers [76].

A cross sectional study done in healthy children in Pakistan showed a low prevalence of anti-HCV of 0.44% [75]. A large community based study of 1997 volunteers done in Karachi by Janjua et al to look at the prevalence of HCV, found that of 1997 participants, 476 (23.8%) were anti-HCV positive [46]. Although the study was a community based study it was done in a particular part of Pakistan and the results may not be representatives of the rest of Pakistan. Nevertheless these data indicate a very high prevalence in this region of the country. A study done in the Punjab province of Pakistan of 1,922 volunteers, showed a high prevalence of chronic HCV infection i.e. anti-HCV 34%(n= 421/1239) in those aged >20years which maybe attributed to the small pox eradication programme .The prevalence in the younger age group was 5.4% (n=37/683) which maybe due to the use of non sterile therapeutic procedures, dental procedures, barber shop shaving and a lack of awareness of the disease[77].

The HCV genotype 3a is the most prevalent genotype in Pakistan. A study looking at 1364 patients at a hospital in Lahore has shown genotype 3a (n=763/1364 -55.9%) to be the dominant genotype[78]. A recent study has shown that in the 189 patients, HCV genotype 3a was the predominant genotype (81.4%) in the cohort studied, followed by 3b (9.3%), 3k (2.3%), 1a (1.5%), 1c (1.5%), 1b (0.8%), and 2a (0.8%) [16].Recent studies done in Pakistan have found an association between HCC and genotype 3a, and of the 82 patients with HCC, 76% were found to be genotype 3a patients.This shows an association between genotype 3 and HCC but this maybe due to the fact that genotype 3 is common in Pakistan [16]. Idrees M et al's group studied 161 patients with HCC and found HCV genotype 3a was the predominant genotype in 40.96% of the patients followed by 3b in 15.66%, 1a in 9.63%, and 1b in 2.40% of HCC tissue samples [15, 16]. These studies have shown that in a high genotype 3 prevalence region HCC is common and therefore associated with genotype 3 because of the high background of genotype 3.

To assess the risk factors associated with HCV transmission in Pakistan, study questionnaires were designed to study potential risk factors associated with HCV transmission in a population based study of 1997 participants in Pakistan. The multilogistic regression analysis showed that those who had received blood transfusions, had dental treatment's in the past, belonged to the province of Sind in Pakistan, were of older age, had a history of multiple therapeutic injection use, extramarital sexual intercourse, history of barber shop shaving, and a history of hospitalisations; were found to be significantly associated with HCV[46]. This study was a large population based study but was done in a specific area of Pakistan, thus the results may not be representative and larger case control studies will be needed to look at the transmission and risk factors associated with HCV in Pakistan.

1.13 Epidemiological patterns of disease transmission

The relationship between HCV spread and the transmission of disease pattern may be divided into three epidemiological patterns [79, 80].

The pattern of disease transmission in developed countries was first identified in the US NHANES study in which 15,079 volunteers were screened and anti-HCV positive persons were 1.3% or 3.2 million people in the United States estimated to be infected with chronic HCV infection. The peak prevalence of HCV i.e. (4.3%) was observed in patients between the age of 40-49 years of age indicating that the transmission of HCV occurred in the past 10-30 years ago with a strong association found with intravenous drug use[43]. In most of the European countries (UK, Sweden, Norway), IVDU has been the dominant mode of HCV transmission during the past 35 years, accounting for 60% to 90% of the chronic HCV infection. In Northern Europe the epidemic was mainly transmitted by IVDU with an overall prevalence between 0.1 and 1%. It was mainly found among adults aged 30–50 years. The reported incidence of acute hepatitis C (2.2 – 9 cases per 100,000 population) was found to be increased in the mid 1990s among those aged 15- 29 years of age, as a result of the epidemic of IVDU that had already started in several Eastern European countries[63, 81].

The second pattern of disease transmission was seen in countries like Japan and Italy. A population based cross sectional study done in north eastern Italy showed that of the 4820 participants 2.4% (n=116/4820) were positive for anti-HCV. The age specific prevalence was found to be low in young adults and was found to be increased in the elderly population. Similarly the Japanese Red cross centres have indicated an age specific prevalence in the general

population of the 272,343 blood donors the HCV prevalence was found to be higher in the older age groups i.e. 1.29% in the 40 to 49 year olds and 2.54% in the 50 to 64 year olds .This suggests that the risk of HCV infection was seen in the distant past i.e. 30-50 years ago. The risk factors which were involved appeared to be unsafe therapeutic injections, use of unsterile needles during acupuncture and use of non sterile equipment during medical procedures and dental treatments [82, 83].

The third pattern of disease transmission is commonly seen in developing countries where HCV infection is prevalent amongst all age groups [84, 85]. There is believed to be an ongoing high risk of acquiring HCV infection due to the continued use of non sterile therapeutic procedures and injection and a lack of awareness of the disease [79, 80].

1.14 Natural History of HCV

The natural history of hepatitis C virus infection is variable and it has the ability to progress over time to cirrhosis and hepatocellular carcinoma. Furthermore, a proportion of those patients who have chronic HCV infection do not die from the disease but from the co-morbid conditions that accompany it. One of the confounding factors that has been a recurrent source of hindrance to the study of the natural history of HCV, is the inability to identify precisely the time of acquisition of infection due to the relatively asymptomatic course of the disease. Presence of elevated aminotransferases for six months or more or the persistence of HCV viraemia for a period of more than six months defines chronic infection, and a proportion of patients clear the virus within the first 6 months of exposure. Chronic HCV infection may last many years before the development of end stage liver disease and HCC which result in the need for liver transplantation.

The ideal method to define the natural history of chronic HCV infection would be to follow up prospectively a large cohort of patients without treatment until the entire group reached a hepatic endpoint or died [4, 86] in the real world this is clearly not possible therefore we can only look at the natural history of chronic HCV infection by analysing the existing prospective and retrospective studies[4].

1.15 HCV-Acute and Chronic Infection

Acute infection with the hepatitis C virus is infrequently diagnosed as the majority of infected individuals remain asymptomatic but occasionally patients with acute HCV infection become jaundiced with raised serum transaminases. A study looking at 60 patients with acute hepatitis C

infection were followed up and it was found that 44% of the patients spontaneously cleared the virus [87]. This showed that there is spontaneous clearance of the virus within the first three months after the onset of symptomatic disease and unnecessary treatment should be avoided [87]. In a study done in a transfusion setting, where 135 patients were followed up after developing acute HCV infection after cardiac surgery, 23% (n=31/135) of the patients spontaneously cleared the infection were followed up for a year whereas 77% (n=104/135) of the patients progressed to chronicity [88]. A prospective study of 41 patients with acute hepatitis C who were followed up for six years found that 24%(n=10/41) of the patients spontaneously cleared the infection 76% (n=31/41) progressed to chronicity [89] and meta analysis of twenty-two studies which had included 1075 patients, showed that the sustained virologic response (SVR) rate for treated patients was 78%, significantly higher than 55.1% in untreated patients (OR = 3.08, 95% CI: 1.8-4.8 P value <0.0001) and the studies indicated a similar rate of persistence of viraemia after acute HCV infection [90, 91] . The onset of symptoms ranges from 3 to 12 weeks after exposure, and may include malaise, weakness, anorexia and jaundice. Serum alanine aminotransferase (ALT) levels begin to rise 2 to 8 weeks after exposure, and often reach values greater than 10 times the upper limits of normal [92].

HCV RNA can be detected in the serum within 1 to 2 weeks after exposure [91]. The level of HCV RNA rises rapidly during the first few weeks, and then peaks shortly before the peak of serum aminotransferase levels and onset of symptoms. In self-limited acute hepatitis C, symptoms can last several weeks and subside as ALT and HCV RNA levels decline followed by the appearance of anti-HCV antibodies and clearance of the virus. Acute HCV infection can be severe, but fulminant liver failure is rare [92].

Chronic HCV infection is marked by the persistence of HCV RNA in the blood for at least 6 months after onset of acute infection. In patients with chronic HCV infection the serum transaminases fluctuate and may vary from day to day hence they may not be considered reliable markers for disease progression. Liver biopsy is considered as the gold standard for the grading and staging of chronic HCV infection, although alternative measures are used to assess the stage of fibrosis i.e Fibro scan, Fibro test, Enhanced Liver Fibrosis test (ELF).

The rate of progression of hepatitis C virus to fibrosis depends on several host or viral factors such as age, gender, consumption of alcohol, duration of infection and viral co-infections [93, 94].

1.16 Cohort studies of HCV acquired from blood or blood products

Irish women who had received HCV contaminated Rh immunoglobulin during 1977 and 1978 were followed up as a part of a national screening program. Of the 704 women identified to be anti-HCV positive, 390(55%) were identified to be HCV RNA positive.Only 2% of these women developed cirrhosis after a period of 17 years [4, 95]. This widely quoted study plays an important role in the study of the natural history of HCV as it involves a large cohort of patients with a known onset of infection. However the study only involved recently pregnant women all of whom have been very carefully reviewed and managed so there may be an element of selection bias as well as modification of the natural history by ongoing medical intervention reducing, for example, adverse factors such as alcohol abuse. In this study only 2% of women developed cirrhosis after 17 years of infection whereas the results from other studies have suggested that 20% of patients with chronic HCV infection develop cirrhosis after 20 years [18].This may be different from other studies as most of the natural history studies have been done in high risk groups i.e. patients from transfusion settings whereas this study involved young women, therefore age of acquiring infection and female gender may have attributed to the slow disease progression in this cohort of patients [4, 95].

Studies from other 'transfusion associated hepatitis' cohorts have helped in long term evaluation of the natural history of HCV. Three large scale transfusion studies were combined where subjects were followed up for 25 years, and found no difference in mortality between cases and controls (the controls were defined as those individuals who had been transfused but had not developed transfusion associated hepatitis) and cases where those individuals who had developed transfusion associated hepatitis. The overall mortality due to chronic HCV infection in this setting was <3% [4, 86, 96]. However patients receiving blood transfusions are inevitably suffering from significant non-hepatic diseases and the failure to observe any change in mortality may have reflected the impact of the underlying disease, rather than any null effect of chronic HCV [95, 96]. However, more prospective longitudinal studies are needed to study the factors affecting disease progression of HCV.

Studies in prospective cohorts infected by contaminated blood products uniformly show a low rate of disease progression. However theses studies have a number of confounding factors that may reduce the impact of HCV infection. A study done in an outbreak of HCV in a plasmapherisis centre in Austria prospectively looked at the natural history of disease

progression in 30 patients over a period of 18 years and found a variable rate of disease progression in patients infected with the same virus i.e. 20% of the patients developed cirrhosis. Increased age at the time of infection was identified as an important risk factor for disease progression [97]. The group found a variable rate of disease progression which maybe attributed to the host factors such as alcohol intake, which may have been increased in this group.

1.17 Studies of patients attending hospital clinics

An alternative approach to evaluating the natural history of HCV is to study patients presenting to liver clinics with HCV. Such an approach has the disadvantage that selection bias may play a significant role in presentation.

A cross sectional study looking at 2235 patients who had undergone a liver biopsy examined the natural history of fibrosis progression in HCV. Fibrosis progression per year was defined as the ratio between the fibrosis stage in METAVIR units and the estimated duration of infection in years. The group looked at 2,235 biopsy samples and duration of infection in patients and found that the rate of fibrosis progression per year was 0.133 fibrosis unit (95% CI 0.125–0.143). However, progression was variable and it was suggested that there are at least three populations of patients: rapid fibrosers, intermediate fibrosers and slow fibrosers. The mean rate of fibrosis progression per year and the course of progression to cirrhosis was not the same for all patients. From the results of the study the estimated time to cirrhosis was 30 years; 33% of patients had an expected median time to cirrhosis of less than 20 years and 31% wouldn't progress to cirrhosis or will not progress for at least 50 years [18].

A systematic review conducted of 111 studies which comprised of (n=33,121) patients with chronic HCV infection found that the estimated prevalence of cirrhosis at 20 years after the infection was 16% and the duration of infection was found to be the most consistent factor associated with progression of fibrosis, showing a nonlinear progression of fibrosis pattern [98].

1.18 Factors that may influence the progression of chronic infection

Various host and viral factors may play a confounding role in the natural history of chronic HCV infection. The presence of one or more factors can have an adverse effect on disease progression.

In a community based prospective study including 1667 patients who had a history of intravenous drug use and were positive for anti HCV, 10% of these subjects spontaneously cleared the infection during follow-up whilst 79% had persistent viraemia. There was a decreased chance of clearing the virus if the patient was male, co-infected with HIV, using

intravenous drugs for more than 15 years, was older than 38 years of age or was from an African American background [86, 99].

A study looking at a 25 year follow up of 310 haemophiliac patients has shown that co-infection with HIV resulted in an increased progression of the disease leading to death in 21% of patients co-infected with the HIV infection [100]. Thus there is a consensus opinion that HIV co-infection accelerates disease progression.

The Irish cohort of 704 women who tested anti HCV positive of those women only 2% developed cirrhosis after a follow up of 17 years and of the women who had developed cirrhosis, 2 of the women were drinking alcohol in excessive amounts and all the women infected were below 45 years of age – this suggests that female sex and age <45 years at the time of infection may be factors which may slow the disease progression [95].

A retrospective analysis of 112 HCV RNA positive patients who had paired liver biopsies showed which that 21% (23 patients) had significant disease progression i.e. >1 Ishak score on the second biopsy and multivariate analysis showed hepatic steatosis to be significantly associated with fibrosis progression [101], suggesting that liver fat may increase fibrosis progression, although whether this is due to factors that may predispose to steatosis (such as alcohol intake) is unclear.

A recent study of 335 treatment naïve patients looking at factors affecting the natural history of fibrosis progression in patients with chronic HCV genotype 3 infections. The study showed that the fibrosis progression rate was higher in patients who acquired infection at > 30 years of age than those < 30 years, and was also higher in those who acquired HCV infection through blood transfusion than with other modes of transmission. The median time to progress to cirrhosis was noted to be 16 years [102]. This rate of progression was similar to that seen in other studies with other genotypes and appears to confirm the widely held view that different genotypes progress at a similar rate [18].

Various modifiable and non-modifiable risk factors associated with fibrosis progression in patients with chronic HCV infection were investigated. Fibrosis progression was determined by studying paired biopsies and their analysis suggested that fibrosis progression over time is not linear and risk factors that accelerated progression included increasing age, increased alcohol consumption and cigarette smoking [103]. Lifestyle modifications may help in slowing the

disease progression in patients with chronic HCV infection and knowing the non-modifiable factors may help in tailoring the patients treatment.

Table 1.2 below shows a summary of the host, viral and external factors that have been implicated in modifying disease progression.

Viral Factors	**Host Factors**	**External Factors**
Viral load	Age at the time of infection:>age	Alcohol
Viral genotype	Sex	Smoking
Viral quasispecies	Ethnic background	Environmental factors
	Co-infection with HBV,HIV	
	Co-morbiditiesi.e. Hemochromatosis, NASH	
	HLA class II antigens	

*Table reproduced from Seeff –Natural History of hepatitis C [4].

1.19 Cirrhosis and HCC

It is difficult to ascertain the incidence of hepatitis C virus infection as most acute infections are asymptomatic thus most of the epidemiologic data are available from prevalence studies. It was estimated by the WHO in 2004 that the annual death rates caused by HCV related liver cancer and cirrhosis were 308 000 and 785 000 respectively [104]. Approximately 20% of HCV patients develop cirrhosis from the infection after about 20 years but it is probable that the prevalence of cirrhosis in patients with chronic HCV increases with increasing duration of infection and after many decades most patients will have developed cirrhosis. Once cirrhosis is established, the rate of progression to HCC is 1% - 4% per year [5].

The prognosis of those with hepatitis C virus related cirrhosis often depends on the development of hepatic decompensation or hepatocellular carcinoma [18, 102]. The 10-year survival of cirrhotic patients with chronic hepatitis C is approximately 50% of those patients with

uncomplicated cirrhosis. Every year about 3-5% of these patients develop hepatocellular carcinoma (HCC) and 1-3% develop decompensated liver disease [102].

Patients with cirrhosis secondary to chronic HCV infection have an increased risk of developing HCC. The major complications of cirrhosis that contribute to morbidity and mortality are sepsis, variceal hemorrhage, refractory ascites, hepatic encephalopathy, severe portal hypertension, renal insufficiency and hepatorenal syndrome. The natural history of cirrhosis secondary to chronic HCV infection has been defined by retrospective and prospective studies which have shown that 80% of patients with well compensated cirrhosis and with no previous episodes of decompensation will survive for the next 10 years whereas once a patient develops an episode of decompensation then their survival declines to 50% after 5 years and 30% after ten years [105, 106]. The prevalence of HCC secondary to HCV related cirrhosis has doubled in the past two decades in the US. In Japan the annual death rate due to HCC secondary to chronic HCV infection is 5% -10% [106] . Prospective studies have suggested an incidence of HCC between 1%-3% in the non-Asian population. The incidence of HCC is greater in African Americans, in males than in Caucasians [105, 106].

The current recommendations are that patients with established cirrhosis should enter a surveillance programme for development of HCC, comprising 6-monthly ultrasound and alpha fetoprotein screening.

MELD score (model for end stage liver disease) has been developed as a marker of the severity of the cirrhosis and is widely used to evaluate the need for transplantation. In the UK a modified version – UKELD is now used, having been developed in 2008. It validates chronic liver disease severity that uses a patient's laboratory values for serum sodium, serum bilirubin, serum creatinine, and the international normalized ratio for prothrombin time (INR) to predict survival. It is calculated by the following formula

5 x {1.5 x loge(INR) + 0.3 x loge (creatinine(µmol/L)) + 0.6 x loge (bilirubin(µmol/L)) - 13 xloge(Na(mmol/L) + 70}

The UKELD was developed following analysis of 1103 patients and was validated in an independent cohort of 452 patients.The minimal listing criteria is that a patient should have a 1 year liver disease mortality without the need for transplantation of >9% which is predicted by a UKELD score of 49 or more. A UKELD score of 60 is predictive of a 50% 1 year survival[107, 108].The aim of developing this system is to have a model for allocation based on the benefit

which takes into account of those people who would have an increasing likelihood of dying without a transplant[108].

1.20 Burden of Disease:

In order to assess the global burden of morbidity and mortality associated with chronic HCV infection the past incidence of acute HCV infection is a major factor which would help in estimating the future prevalence of disease. In the United States national seroprevalence data (NHANES) and age specific incidence data were put together to look at the future prevalence of disease. The model showed that the highest incidence of infection was between the 1960's to 1980's with a decline in the 1990's projections showing that the decline in prevalence of chronic HCV infection could be due to the decline in incidence of the disease in the 1990's and the number of persons who would be infected for >20 years and that there would be a peak incidence of HCV by the year 2015 [109]. To estimate the burden of disease in Europe, literature and health databases were searched for HCV specific burden of disease data and it was estimated that 7.3-8.8 million people (1.1-1.3%) were infected with chronic HCV infection and a quarter of liver transplants performed in 2004 were due to chronic HCV infection [110]. However, a more stringent strategy is needed to estimate the prevalence and incidence of chronic HCV infection in Europe. In order to assess the burden of disease due to chronic HCV infection, large prospective community based studies are needed as the data that is used to model the disease in Europe comes from high risk populations which would not tell us the true prevalence of the disease in that region [111]. Prospective community based studies are needed in developing countries e.g. Egypt, Pakistan, Africa where the prevalence of chronic HCV infection is endemic and the true prevalence of disease is still not known.

1.21 Modes of Transmission

Hepatitis C is a blood borne virus that is mainly transmitted through blood contact. It is common in those who have used intravenous drugs, in those who shared injecting paraphernalia or those patients who had received contaminated blood or blood products prior to early 1990's [111]. Sexual transmission of HCV is uncommon and materno-fetal transmission is rare.

A retrospective review of 559 live born infants studied has shown that of the 441 infant records reviewed 18 (3.2%) tested positive for HCV giving an overall prevalence of 4.1% (18/441, 95% confidence interval 2.3%-5.9%) .The rate of HCV in infants born by vaginal delivery was 7.2% (17/236) those born by ceaserian section was 5.3% (1/19) ,this difference was not statistically

significant. There was no difference seen in the rate of vertical transmission of HCV and the mode of delivery [112]. In a study involving 441 mother child pairs it was found that of the 227 births materno-fetal transmission was present in overall 6.7% of the cases with a vertical transmission rate 3.8 times higher in HIV co-infected women, this was found to be statistically significant (p=0.06). However no effect of transmission of HCV was seen in babies that were breast feeding [113]. Another study has shown an overall rate of vertical transmission of HCV 5% (n=13/275 infants) [114]. Several studies of a similar size have indicated a similar level of transmission however these studies were conducted mostly in Caucasians and in developed countries where the modes of transmission are reduced as compared to the developing countries. In the developing world few studies have been done but a study done in 499 healthy Egyptian pregnant women showed that anti-HCV was present in 13% of the women with an overall seroprevalence of 4.3%. Of the 20 women who had tested positive for HCV there was a 100% materno-fetal transmission (n=20/20), this study showed a significantly high materno-fetal transmission rate (5%; p=<0.0001) [115]. The transmission rate was higher in this study as compared to the large studies but this study was done in a country where HCV is endemic and the risk of materno-fetal transmission may be increased significantly. In a retrospective study done in co-infected pregnant women in which 62 infants were born to 54 HCV and human immunodeficiency virus (HIV), co-infected women were enrolled in a prospective study of HIV transmission. The overall rate of vertical HCV transmission was 16.4% (9/62). Most HCV infected children did not develop antibodies to HCV. The rate of HCV infection was higher among the HIV co- infected infants (40%) than among HIV uninfected infants (7.5%; odds ratio, 8.2; p =<0.009) [116]. This shows that the rate of vertical transmission of HCV is higher in the co-infected patients but more larger prospective studies are needed.

In the developing countries HCV infection can be acquired due to the use of unsterilised medical equipment, dental procedures, barber shop shaving and blood transfusion. The routes of transmission of HCV in Pakistan can range from the re-use of glass syringes/needles, lack of trained medical staff in the transfusion and dialysis setting, IV drug abuse, and men who have sex with men. In recent years there has been an outbreak of acute and chronic HCV infection in men who have sex with men. The factors leading to HCV transmission are not clear but a community based study looking at the incidence of sexual transmission of HCV in HIV positive and HIV negative homosexual men, found that traumatic anal sex was associated with a high rate

of transmission and HCV prevalence was found to be 1.07% in the HIV negative cohort and 9.39% in the HIV positive cohort [117, 118]. Sexual transmission of HCV was uncommon in the general population but was found to be more common in IVDU's and HIV positive homosexual men. A study looking at the prevalence of HCV in north eastern Thailand has shown that of the 3,255 volunteer blood donors antibodies to HCV were detected in 6.5% of male blood donors and 0.9% of female blood donors, giving an overall prevalence of 5.6% in this population[119]. Effective blood screening in blood donors for anti-HCV antibody is necessary in developing countries to help in limiting the spread of HCV. For example in a study of 523 volunteers in Pakistan showed that there was a higher seroprevalence of anti-HCV in individuals vaccinated for smallpox versus nonvaccinated individuals (21.0% vs. 4.6%, $P < 0.001$, odds ratio, 3.39; 95% confidence interval, 1.36-8.46) [77]. A number of groups have suggested that smallpox vaccination may be associated with chronic HCV infection but larger studies are needed to study the history of small pox vaccination and the endemic spread of HCV [73, 77] . In the developing world HCV transmission is through unsafe therapeutic injections, other healthcare procedures and through unscreened transfusions [73, 77, 120].

1.22 History of HCV therapy – Combination Therapy

The combination of pegylated interferon alpha (2a or 2b), given once a week, plus ribavirin, taken daily, is the standard of care for chronic hepatitis C infection, although newly released directly acting antiviral agents may modify this. The aim of treatment is to attain a sustained virological response (SVR) which is defined as undetectable HCV RNA levels 24 weeks after cessation of therapy. The six genotypes of HCV virus respond differently to treatment. About 80% of patients infected with genotypes 2 and 3 respond to treatment whereas patients with genotypes 1, 4, 5 and 6 have a 40% -50% response rate to therapy [121]. Ribavirin is an oral antiviral agent that has activity against a broad range of viruses. By itself, ribavirin has little effect on HCV, but given in conjunction with pegylated interferon it increases the sustained response rate by two to three-fold over that of interferon alone. Patients with an SVR have a 95% chance of being virus free 5 years later, regression of fibrosis progression, decreased incidence of hepatocellular carcinoma and an overall decrease in morbidity and mortality [121].

The recommended duration of therapy for HCV genotype 2 or 3 infected patients is 24 weeks of combination therapy with pegylated interferon and ribavirin while patients infected with

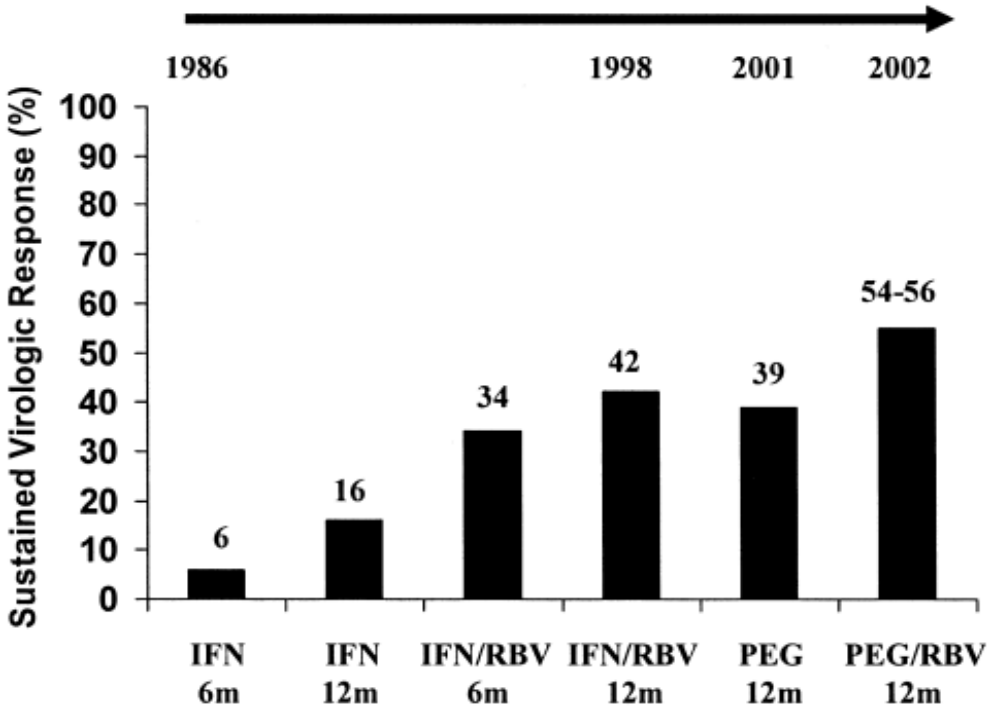
genotype 1 and 4 should receive 48 weeks of combination therapy. The current recommendations state that if a patient with genotypes 1 and 4 chronic HCV infection fails to achieve a 2 $\log_{10}$ drop in viral load by 12 weeks of treatment, consideration should be given to stopping treatment as achieving an SVR may be unlikely. There is no recommendation for genotype 2 or 3 patients to measure HCV RNA at week 12 given the high response rates [122, 123]. The dose of ribavirin also influences the SVR. The percentage of patients who had an SVR increased from 40% to 50% when the ribavirin dose was increased from 12 to 16 mg / kg in patients with genotype 1 infection, but a similar effect was not seen in genotype 2 and 3 patients [123]. Studies have suggested a beneficial effect with the use of pegylated interferon monotherapy for 24 weeks in patients with acute hepatitis C in order to prevent the progression of disease and to avoid the development of chronic infection [91, 124].

1.23 Clinical trials:

Hoofnagle JH et al first suggested that interferon may be effective in the management of hepatitis C. In his preliminary report, treating patients with non-A non-B hepatitis with human alpha interferon given daily to 10 patients resulted in normalisation of serum aminotransferases and it was seen that treatment resulted in improvement in the liver histology, further prospective randomised controlled studies were clearly indicated to look at the effect of interferon in the treatment of non-A non-B hepatitis[125]. Poynard et al studied the effect of long term treatment for 18 months in 329 patients with chronic non-A non-B hepatitis and found that thrice weekly treatment with interferon alpha 2b resulted in an improvement in liver histology and serum alanine amino transferase [126]. Back at that time quantitative and qualitative measurement of viral load were not available in these patients but there was a clue that interferon could be used for the treatment of chronic non-A non-B hepatitis. The initial treatment of chronic HCV infection was monotherapy with interferon alfa-2b which produced a SVR of 15%-20% when given for 24 – 48 weeks [127] . However when this drug was combined with the antiviral agent ribavirin (of little value as monotherapy [128]), it produced a dramatic increase in response rate of 31%-41% in patients with chronic HCV genotype 1 infection [128, 129]. Longer lasting interferon's involving chemical modification with the addition of a polyethylene glycol moiety were then introduced after the introduction of ribavirin and randomised control trials of combination treatment with ribavirin and pegylated interferon-alfa 2b produced a response rate

of 40%-50% in genotype 1 patients and about 80% in patients with chronic HCV genotype 2/3 infection [129]. Peginterferon has an extended serum half life which helps to provide constant viral suppression for seven days, allowing a once weekly dosing regimen. This has proven beneficial for patients as once weekly subcutaneous injection rather than the alternate day injections resulted in an improvement in patient compliance.

Two forms of pegylated interferon are now available ie pegylated interferon alfa -2b is administered as a weight based regimen whilst pegylated interferon alfa 2a is given at a fixed dose of 180mcg once weekly. The graph below shows the different interferon regimens adopted over time with their respective response rates.



- Figure1.2 History of HCV Treatment Regimes(reproduced from AASLD guidelines).[130]

In large comparative randomized controlled trials comparing treatment regimens i.e. combination therapy of peginterferon alfa-2a and ribavirin , peginterferon alpha 2a monotherapy and peginterferon alfa-2b and ribavirin, it was shown that combination treatment of peginterferon alfa 2a and ribavirin was superior than other treatment regimens, with a sustained response of 56% as compared to 44% in patients treated with interferon alfa 2b and ribavirin [131]. The IDEAL study compared the effect of pegylated interferon alfa 2a versus pegylated interferon alfa 2b in different doses (pegylated interferon alfa 2b 1.5mcg/kg and pegylated interferon alfa 2b 1.0 mcg/kg) with weight based ribavirin dosing and flat/fixed dosing of ribavirin in patients with genotype 1 chronic HCV infection and found that the overall SVR was similar in all the three treatment arms (40 vs. 38 vs. 41 percent, respectively) [132].

1.24 Response guided treatment in Genotype 2 and 3 infected patients

Dalgard et al looked at shortening treatment duration in genotype 2 and 3 patients with the help of rapid virological response guided treatment. RVR is defined as an undetectable HCV RNA at week 4 of therapy. RVR guided therapy in a randomised control trial of 428 patients of 14 versus 24 weeks of combination therapy with pegylated interferon alfa2b and ribavirin (800mg-1400mg). If the patients week 4 virological response was >50 IU/ml they were allocated the 24 week arm and if their week 4 HCV RNA <50 IU/ml then they were allocated to either the 14 week treatment arm or the 24 week treatment arm. Of the 343 genotype 3 patients enrolled in the study 119 patients were allocated to the 14 week arm and 224 patients were allocated to the 24 week arm. Overall SVR in the 14 week treatment arm was 84% (n=93/110) and in the 24 week treatment arm was 92%(n=106/115).The overall relapse rate was 16%(n=17/110) in the genotype 3 patients randomised to the 14 week treatment arm than the 24 week treatment arm 8%(n=9/115) [133].The study did not define patients with respect to different ethnicities ,whether the patient were cirrhotic or not and an APRI score was used to define the fibrosis progression in the patients rather than a liver biopsy. This study was relatively small and may have been underpowered to detect a true difference with the longer treatment duration.

In a larger, fully powered study involving PegIFN alpha 2a Shiffman et al showed that 16 weeks of treatment was inferior to 24 weeks of treatment in patients with chronic HCV genotype 3 infections. The SVR in the 16 week arm was 62% (221/358) as compared to the 24 week

treatment arm 66% (244/369).RVR guided therapy has been recommended for genotype 2 and 3 patients to shorten treatment duration on the basis of attaining an RVR but in this study in the 16 week treatment arm 64% (n=230/358) and in the 24 week arm 59% (n=219/369) patients achieved an SVR. The study has shown that patients should not be treated for less than the recommended duration of 24 weeks [134]. Thus two studies have shown slightly different responses in patients with genotypes 2 and 3 HCV exposed to shorter durations of therapy. A number of studies from Italy (Mangia et al) confirm the original Dalgard observation suggesting that shorter treatment durations are effective but all of these studies have been relatively small. It is noteworthy that studies demonstrating the benefits of shortened durations of therapy have used PegIFNalpha 2b and have been relatively small scale and the only study showing no benefit from shorter durations of therapy used PegIFNalpha 2a but was a larger multicentre study. It is therefore unclear whether the observed differences are genuinely due to differences the two preparations of interferon or whether the differences represent inadequate power in some of the smaller studies. Of note for this thesis is the fact that these studies included very few patients with cirrhosis or advanced fibrosis and the optimal duration of therapy for patients with advanced fibrosis is not clear.

It has been suggested that the cause of relapse or treatment failure in patients with genotype 2 and 3 HCV may be due to the use of lower dose of ribavirin or due to dose reductions in ribavirin. Note that PegIFN alpha 2b is normally combined with a higher dose of ribavirin in patients with genotype 2 and 3 HCV (800–1400 mg depending upon body weight) whereas PegIFN alpha 2a is always used with a flat, fixed dose of ribavirin (800mg). However the impact of ribavirin dosing was not examined in these studies. Likewise the impact of RVR on treatment, the impact of genotype 3 and cirrhosis were not examined.

Many studies have focused on shortening treatment duration in genotype 2 and 3 patients due to the high SVR rate of 80% achieved with a 24 weeks of treatment. It is important to recognise that genotypes 2 and 3 respond differently to treatment and both genotypes have different treatment outcomes. The ACCELERATE study (discussed above) which has compared 16 weeks of treatment versus 24 weeks of treatment in genotype 2 and 3 patients found a significantly higher relapse rate in the 16 week group (31%) as compared to the 24 week group (18%; $P<0.001$) thus shorter treatment duration was associated with a higher risk of relapse. The study showed an overall reduced response rate in genotype 3 patients when compared to genotype 2

patients [134-137]. The WIN-R trial also demonstrated that genotype 2 patients have a higher SVR rates and lower relapse rates (72% and 5%, respectively) as compared to the genotype 3 infected patients (63% and 5% respectively) [123, 138]. An Indian study compared two different dosing regimens of pegylated interferon alpha 2b in combination with ribavirin given for 24 weeks in genotype 3 patients and showed that peginterferon alfa 2b at 1.0 mcg/kg/week with ribavirin is as effective as peginterferon- alfa 2b at 1.5 mcg/kg/week with ribavirin in patients with chronic HCV genotype 3 infection [139]. The table below shows the comparison between different treatment durations and patterns of response in genotype 2 and 3 patients[140].

Table 1.3 Summary of Studies done in Genotype 2/3 Patients

Table1.3 reproduced from AASLD guidelines showing summary of studies done in Genotype 2/3 [140]

Summary of Studies Comparing Short Versus Standard Therapy Stratifying Based Upon RVR in Genotype 2 and 3 Patients

Trial/ Regimen	**PegIFN α-2b 1 μg/kg/wk & Rbv 1,000-1,2000 mg daily**			**PegIFN α-2a 180 μg/wk & Rbv 800-1,200 mg daily**			**PegIFN α-2a 180 μg/wk & Rbv 1,00-1,200 mg daily**		**PegIFN α-2a 180 μg/wk & Rbv 800 mg daily**	
N	283			153			150		1,469	
Gt 2	76%			26%			100%		50%	
Gt 3	24%			74%			0%		50%	
Rx duration	12 wks	24 wks	24 wks	16 wks	24 wks	24 wks	16 wks	24 wks	16 wks	24 wks
n	113	80	70	71	71	11	50	100	732	731
RVR	100	0	64	100	100	0	86	87	67	64
ETR	95	68	79	94	85	72	100	98	89	82
SVR	85	64	76	82	80	36	94	95	62	70
REL	9	6	4	13	5	50	6	3	30	13

[a] Patients were randomized at baseline to a standard 24 week regimen (Group III), or a variable-duration regimen depending on results of HCV RNA testing at week 4: HCVRNA negative-treatment duration 12 weeks (Group I) or HCV RNA positive-treatment duration 24 weeks (Group II).

[b] All patients treated for 4 weeks, patients with an RVR (HCV RNA < 600 IU/ml) were randomized to 16 (Group 1) or 24 weeks (Group 2). Patients with HCV RNA ≥600 IU/ml were treated for 24 weeks (Group 3).

[c] Patients randomized 1:2 to either 16 or 24 weeks.

[d] Patients randomized to 16 or 24 weeks. Abbreviations: Gt, genotype; n, number; Rx, Treatment; REL, Relapser.

1.25 RESPONSE GUIDED THERAPY FOR GENOTYPE 1

A rapid virological response (RVR), is defined by a 2 log drop in viral load or an undetectable HCV RNA at week 4 of therapy, these HCV genotype 1 patients are highly responsive to PEG-IFN plus ribavirin therapy and have a good probability of achieving SVR (rates ranging from 80% to 90%)[141].A metaanalysis of 7 RCT of 807 patients has shown that 397 patients received a shorter than 48 week treatment duration and 410 patients were treated for 48 weeks.The metanalysis has shown that shortening treatment duration to less than 48 weeks cannot be applied to all HCV genotype 1 patients who have attained an RVR as there was a higher rate of relapse in the shorter treatment group. However in the three studies, involving 212 patients, in the subgroup of patients who had a low baseline HCV-RNA level (<400,000 IU/ml) and a RVR, there was no significant difference found between 24 and 48 weeks of PEG-IFN/ribavirin therapy, suggesting that 24 weeks of therapy is the appropriate treatment duration in this group of HCV genotype 1 patients [142].

Early virological response is defined as a 2 log drop in HCV RNA at week 12 of treatment. Genotype 1 patients who fail to achieve an EVR have been recommended to have their therapy discontinued due to the very low probability of subsequently achieving SVR (the "stopping rule") whilst there is no stopping rule for the other genotypes. The predictability of SVR based on EVR was assessed in various studies. Davis et al looked at the effect of EVR on SVR and found that 63% (n=144/229) of the genotype 1 patients who had achieved an EVR achieved an SVR [131, 143].

Patients who do not achieve a 2 log drop in HCV RNA level by week 12 or those who remain HCV RNA detectable throughout treatment are classified as non responders. Studies have looked at different treatment regimens for this group of patients. The first phase in the viral kinetics in response to combination therapy is a rapid dose-dependent decline in viral load. The second phase of viral decline in non responders shows a much slower decline as compared to the responders. A first phase decline can be observed in almost all patients treated with interferon-alpha, but it was found that the non-responders revealed no further decline of viraemia during the second phase. Studies have shown a similar first phase in non responder patients and patients who respond to therapy and have shown that there is minimal reduction in viral load with continuing therapy [144, 145]. The REPEAT trial which was done in 950 patients showed an overall improvement in SVR rates in previous non responders when given an extended duration of therapy. The overall rate of SVR after retreatment was found to be lower in non responders than in patients who have relapsed [146, 147].

Relapse is defined as the reappearance of HCV RNA after discontinuation of therapy. The EPIC-3 study showed an overall SVR of 43%(n=130/300) after retreatment of patients who had relapsed previously while the overall SVR of 55%(n=35/64) was seen after re treatment for 48 weeks in patients who had relapsed after 24 weeks of combination therapy .A recent metanalysis of 7 RCT has shown a higher rate of relapse when treatment duration was shortened in HCV genotype 1 patients [142, 146, 148, 149].

Null response is defined as a <1 log $_{10}$ drop in HCV RNA during the course of treatment. The HALT-C trial which comprised of 1,050 patients and the REPEAT study have looked at baseline factors which are associated with predicting null response (including genotype 1 infection, African American race, older age, previous therapy) and the on treatment predictive factors, which include cytopaenias, smaller reduction in ALT, less decrease in body weight, less decrease in haemoglobin and platelets) [146].

1.26 Protease Inhibitors

It has been shown in several studies that treatment with PEG-IFN-alpha/RBV fails in approximately 60% of patients with chronic HCV genotype 1 infection. Direct acting antiviral agents, protease inhibitors, boceprevir and telaprevir in combination with PEG-IFN alpha/RBV have resulted in significantly improved SVR rates in treatment naıve and previously treated patients. In previously treated patients they lead to significantly higher response rates than are

seen with pegylated interferon alfa and ribavirin alone. The PROVE 3 trial was a phase 2b clinical study which enrolled 453 genotype 1 HCV infected previous treatment failure patients. The highest SVR rate was observed in the 24 week triple therapy arm with telaprevir, followed by additional 24 weeks of PEG-IFN and ribavirin. Realize was a phase III clinical study which enrolled 662 treatment experienced patients (i.e. previous non-responders, partial responders or relapsers; patients with previous viral breakthrough were excluded).The response rates were found to be higher in the Telapravir containing arm however patients with genotype 1b responded better to treatment than 1a. Thus Clinical studies have demonstrated significantly better SVR rates with triple therapy of telaprevir + PEG-IFN alpha/RBV compared to PEG-IFN-alpha /RBV alone in treatment-experienced patients.

In treatment naïve patients protease inhibitors also enhace response rates. The ADVANCE study (n=1088) was a randomized placebo controlled trial designed to compare 8 weeks versus 12 weeks of telaprevir triple therapy (with PEG-IFN/RBV) followed by either 12 or 36 weeks of PegIFN-alpha 2a plus ribavirin. The conclusion of the ADVANCE study was that 12 weeks of telaprevir was superior and preferable to 8 weeks and both regimes were significantly better that previous standard of care. The ILLUMINATE study was a randomised controlled trial designed to compare 24 versus 48 weeks of treatment in patients with an eRVR (i.e patients who were HCV RNA undetectable at week 4 and who continued to have undetectable HCV RNA until week 12) .The ILLUMINATE study has shown that patients with an eRVR should be treated for only 24 week.Telapravir is initiated along with PEG-IFN/RBV for the first 12 weeks of treatment. Patients are then continued with PEG-IFN/RBV for a total of either 24 or 48 weeks [2, 150, 151] depending upon the early virological response.

The SPRINT-2 study (n=1097) was a randomised, double-blind, phase 3 trial that compared safety and efficacy of combination treatment with pegylated interferon alpha plus ribavirin (the previous standard of care) with or without boceprevir (800 mg three times a day with food) in ethnic groups of previously untreated black and non-black patients with genotype-1 chronic HCV infection.The SVR rate was found to be higher in the Boceprevir treatment group. Findings of this trial resulted in a response guided therapy recommendation for boceprevir and the complex dosing regimen is shown in the table below[2, 150].Ethnicity and response to treatment need to be looked into independently as the number of black patients enrolled in the studies was very small.

However there is only minimal evidence of benefit of efficacy of protease inhibitor use in non genotype 1 patients. Therefore, neither boceprevir nor telaprevir should be used in patients with non genotype 1 HCV infection [152].

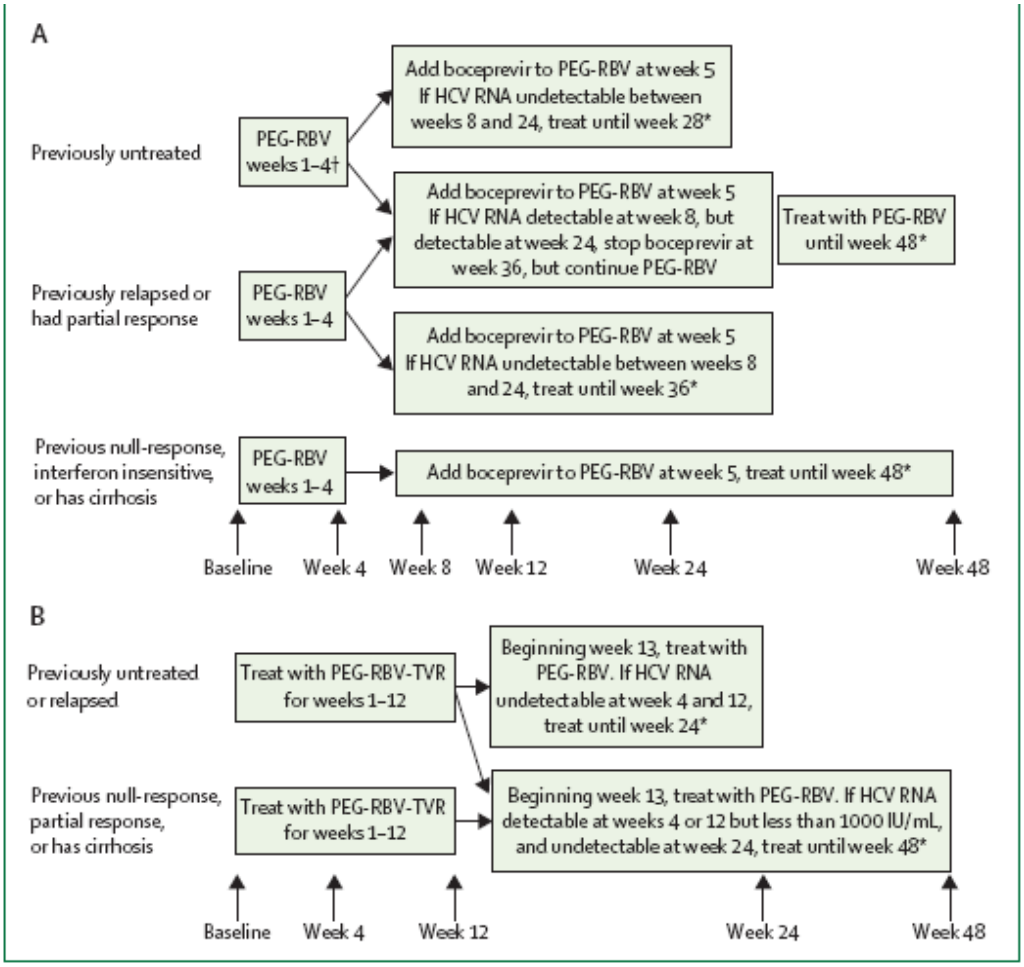


Figure 1.3: Showing Dosing regime of the Protease inhibitors

(Reproduced from the review by Pearlman)[2]

1.27 Use of different dosing regimes of combination therapy HCV genotype 3 patients

The WIN-R trial looked at different doses of ribavirin with pegylated interferon alpha2b and compared treatment durations i.e. 24 weeks versus 48 weeks. In the 802 genotype 3 patients the SVR rates were found to be 70% and 72% in the fixed dose and weight based ribavirin dose and there was no difference seen in SVR rates between the 24 week and the 48 week treatment arm.

Although the relapse rates were 10.6%(n=24/226) and 12%(n=23/191) in the fixed dose and weight based dose of ribavirin[153].The study showed that there was no benefit of extending treatment duration in genotype 2/3 patients and 24 weeks of treatment and a flat dose of ribavirin was found to be adequate.The study included cirrhotic genotype 2/3 patients but data on genotype 3 patients with cirrhosis and how they responded to therapy were not reported. Differences in relapse rates between 24 weeks or 48 weeks of therapy and how treatment could be tailored in the cirrhotic genotype 3 patients was not presented.

Abergel et al looked at patients with advanced fibrosis i.e. F3 and F4 according to the METAVIR scoring system in a multi centre randomised controlled trial comparing two different doses of pegylated interferon alpha 2b with ribavirin for 48 weeks. Of the 58 genotype 3 patients with advanced fibrosis that were included in the study 30 received standard dose of pegylated interferon alpha 2b and 28 patients received low dose pegylated interferon alpha 2b,the SVR for both the groups were similar i.e. 73% in both groups thus the study has shown that patients with advanced fibrosis/cirrhosis can benefit from a lower dose of peginterferon when given treatment for 48 weeks [154]. On the contrary a study done by Hadziyannis S et al found no difference in SVR rates by extending treatment duration i.e. 24 weeks or 48 weeks in genotype 2/3 patients. The study included 288 genotype 3 patients and 90 patients with cirrhosis were included in the study.The study did not define how many of the genotype 3 patients had cirrhosis and the study combined data from genotypes 2 and 3 patients [155]. A larger prospective study is needed to study treatment duration and the effect of different doses of ribavirin in genotype 3 patients with advanced fibrosis and cirrhosis as the retrospective analyses reported above do not adequately address this issue.

Ferenci et al looked at the different doses of ribavirin i.e. 400mg versus 800mg in combination with peginterferon alpha 2a in genotype 2 and 3 treatment naïve patients. Of the 192 treatment naïve genotype 3 patients included in the study, the SVR in the group receiving 400mg of ribavirin with peginterferon alpha 2a was 67.5% as compared to 63.9% patients receiving 800mg of ribavirin with peginterferon alpha 2a.The rate of relapse was 17% in patients treated with 400mg of ribavirin with peginterferon alpha 2a and 20% in patients treated with 800mg of ribavirin with peginterferon alpha 2a thus there was not much difference seen in SVR and relapse rate between the two groups of patients. Those patients who had achieved an EVR 75% of those patients had achieved an SVR [156].

1.28 Factors that influence response rate

Studies have suggested that there is an improvement in response rates, with a lower rate of relapse, in genotype 1 patients with a higher body weight when treated with higher dose of pegylated interferon and ribavirin. A decrease in relapse rate was seen in the higher dose group when compared with the standard dose group (19% versus 40% respectively) [157]. The WIN-R trial has also suggested that weight based ribavirin dosing is more effective than fixed dosing in combination with pegylated interferon alpha 2b in African American genotype 1 patients, resulting in a better SVR rate with lower rates of relapse [158].

Studies have shown that genotype 1 patients who are slow responders can achieve an SVR by extending the treatment duration from 48 weeks to 72 weeks, resulting in an increase in the SVR in the latter group [159-161].

Predictors of response to treatment include African American ethnicity, genotype 1 infection and high viral load (>2 x 10^6 copies/ml, equivalent to 800,000 IU/ml). Such patients have a low rate of SVR as compared to Caucasian patients with genotype 2 or 3 infection with a low viral load. In recent years retrospective review of data from the pivotal clinical studies has been used to develop management guidelines based upon an assessment of the virological response (Response Guided Therapy). Viral kinetics are monitored closely during antiviral therapy and are used to tailor therapy to the individual patient [130, 140].

1.29 Ethnicity and response rates

The prevalence of chronic HCV infection is higher in African Americans (3%) compared to Hispanics (1.3%) and African Americans are less likely to respond to combination treatment with pegylated interferon and ribavirin than Caucasians [162]. Several studies have shown that African Americans have a lower response to therapy and have a higher prevalence of HCC as compared to Caucasians. In a retrospective analysis of 302 patients showed that African Americans had lower fibrosis scores as compared to Caucasians and this was significant in multivariable analysis [163, 164].

This group is known to have an overall lower response rate than other ethnic groups and have a higher incidence of HCC. Conjeevaram et al compared response rates between 196 African Americans patients and 201 Caucasians patients who were treated with combination therapy for 48 weeks. This study was done in 401 genotype 1 treatment naïve patients and showed that the African Americans had lower overall rates of SVR (28%) as compared to Caucasians (52%)

[162]. Furthermore, another study has shown that black race was the only significant variable associated with a reduced response rate to antiviral therapy [165]. A retrospective study of 376 patients which compared response rates in 42 genotype 2 and 3 African American patients versus 334 Caucasian patients showed a decreased response rate (57% versus 82%) suggesting that African Americans have an overall reduced response to combination treatment with pegylated interferon and ribavirin [166].

Genome wide association studies done by Ge et al in 1,600 genotype 1 patients of the IDEAL trial looked at why patients from the European ancestry do better than patients from African American background. It was found that a polymorphism of the C allele at a locus upstream of the IL-28 gene led to a better response to treatment with pegylated interferon and ribavirin [167]. Another genome wide association study was done in genotype 1 patients to look at the association of SVR to combination therapy with pegylated interferon and ribavirin in 293 Australian patients and 555 replication cohort patients and the association with the IL-28 genotype was confirmed [168]. All the IL28B genome wide association studies to date have been done in genotype 1 patients and prospective studies need to be done on patients with other genotypes and from different ethnicities.

Published data has shown that Asian ethnicity is associated with a reduced response rate to treatment. In one study, 16 of 38 genotype 3 (42.1%) Asians who originated from the Indian subcontinent achieved a SVR, compared with 41 of 66 (62.1%) Caucasians ($p = 0.063$)[169]. This study was done on a small number of patients and needs to be validated on a larger cohort of patients to determine whether or not Asian ethnicity is associated with a reduced response rate.

1.30 Treatment in HCV Genotype 3 : Factors affecting response rates

The current standard treatment recommended for hepatitis C genotype 3 patients is 24 weeks of pegylated interferon and ribavirin [134]. In treated chronic HCV patients a number of different virological responses have been reported. These include early virological response with loss of virus after a few weeks therapy; late virological response with loss of virus after several months; relapse whilst on therapy where a virological response is not sustained during ongoing therapy and virological relapse where virological response at the end of therapy is not sustained.

HCV genotype 3 is considered easy to treat. The pivotal trials show a response rate of 80% in patients with chronic HCV genotype 3 infection [129]. However studies from Birmingham have

shown an overall SVR 64.42%(n=67/104) with a reduced response in Asian patients 41%(n=16/38) as compared to the Caucasians 62.1%(n=41/66) this was found to be statistically significant (p=0.063) on a univariate analysis[169] .This study differed from pivotal studies as it examined response rates in patients originating from the Indian subcontinent as all the big studies have looked at patients who originate from Fareast Asia i.e. China, Vietnam, Korean patients with only 16% originating from India and 1.6% originating from Pakistan. However as the numbers were very small and nothing was found to be significant in a multiregression analysis no definite conclusion was drawn from the study and a larger studies would be needed to look at whether there is a difference in response to treatment between Asians and non Asians. However unpublished audits in East London have also shown that patients from the Indian subcontinent who were infected with HCV genotype 3 respond poorly to therapy with a response rate of only 50%. It is not yet clear why the response rate in these patients is so poor but possible factors include the presence of advanced liver fibrosis, increasing age, concomitant diabetes as well as unidentified ethnic and virological factors.

There is evidence that patients with HCV genotype 2 or 3 and with a higher baseline viral load (>800 000 IU/ml)[136, 137] and lower pre-treatment ALT have lower rates of SVR and higher relapse rates after 24 weeks of treatment than those with lower baseline levels of HCV RNA and higher baseline ALT [123]. In addition those patients who have a detectable HCV RNA at 4 weeks of treatment have a lower chance of attaining a sustained viral response .Data from the WIN-R trial, involving treatment with pegylated interferon alpha-2b with two different doses of ribavirin, showed that there were higher SVR rates and lower relapse rates in genotype 2 infected patients compared with genotype 3 (72% vs. 63%, and 5% vs. 10%, respectively)[123, 170]. A study done by Aghemo et al suggests that patients infected with genotype 3 who have cirrhosis are 10 times more likely to relapse following treatment with conventional or peginterferon plus ribavirin than those without cirrhosis[171]. It is possible that genotype 3 infected patients would benefit from longer treatment duration and/or higher ribavirin doses however current data supporting this comes predominantly from retrospective analyses and requires evaluation in prospective clinical trials.

1.31 Extra-hepatic manifestations

Patients with chronic HCV infection may present with various extra hepatic manifestations or syndromes which are considered to be of immunologic origin, including cryoglobulins. These are

detected in the serum of about one-third of patients with hepatitis C virus infection, but the clinical features of essential mixed cryoglobulinemia develop in only about 1–2 percent of patients [103, 128].

Chronic HCV infection may also be a major underlying cause of porphyria cutanea tarda[172].

Since the discovery of hepatitis C virus in 1989, chronic infection with HCV has been associated with the development of type 2 diabetes. The major risk factors for developing diabetes are older age, obesity, HCV genotype 3, liver and kidney transplantation, a family history of diabetes and severe liver fibrosis[173].Treatment with interferon leads to an improvement in glucose tolerance (Konrad,Vicini et al,2000). Other studies suggest that interferon may induce islet cell antibodies (Piquer et al,2001) and interferon can cause insulin resistance in visceral and peripheral tissues of patients with HCV (Imano et al.,1998).Thus the role of HCV and/or interferon therapy in the development of diabetes remains controversial.

1.32 Re-treatment of Chronic HCV infection

The current problem that has been seen in the treatment of patients with chronic HCV infection is relapse or non response to the current standard of care. It was shown in the REPEAT trial that extending treatment duration to 72 weeks from 48 weeks in previous non responders resulted in a SVR of 16% as compared to 8% in the patients on the 48 week arm [146].

Re-treatment of patients who previously relapsed following treatment with historic interferon regimens was also an important issue, as those patients were more likely to achieve an SVR when retreated with pegylated interferon and ribavirin. The EPIC-3 study has shown an overall SVR of 43% (n=130/300) after re-treatment of patients who had previously relapsed after conventional interferon and ribavirin [148]. Regarding re-treatment of those patients who had relapsed after receiving inadequate duration of treatment (i.e. 24 weeks or less), this patient group may benefit from extending treatment duration up to 48 weeks. Randomised trials are needed to confirm the theory of extending treatment duration in previous relapsers.

Several long term trials have looked at the significance of maintenance therapy in previous non responders and patients with advanced fibrosis. The HALT-C trial which comprised of maintenance therapy of low dose interferon (90mcg of peginterferon alpha 2a for 3.5 years) showed that maintenance therapy did not reduce the incidence of long term complications (cirrhosis, HCC, or a decrease in morbidity and mortality) in patients with advanced fibrosis who had previously not responded to standard treatment[174] . The COPILOT study is another

maintenance study which compared the effects of long term low dose peginterferon alpha 2b (0.5 mcg/kg weekly) with colchicine in previous non responders with advanced fibrosis for a period of 4 years. No significant difference was seen between the two treatment groups in terms of long term complications [175].There proved to be no significance of long term maintenance therapy in the difficult to treat patient groups i.e. non responders, cirrhotics.

1.33 Adverse Reactions with Treatment

Peginterferon plus ribavirin is used as the standard of care for treatment of patients with chronic HCV infection. The use of interferon-alpha with or without ribavirin is found to be associated with a range of adverse effects, including influenza-like symptoms, thyroid disorders i.e. hypothyroidism, hyperthyroidism, alopecia, haematological changes i.e. hemolytic anemia, thrombocytopenia, neutropenia and neuropsychiatric disturbances i.e. depression, suicidal ideation and mood swings. Depending on the severity of the adverse events, dose reduction and discontinuation from treatment is frequently seen in patients undergoing therapy[129, 134].

1.34 Mechanism of action of Interferon

Interferon has antiviral, antiproliferative and immunomodulatory activities. Interferon alpha has potent antiviral activity but does not act directly on the virus or its replication complex, instead acting by induction of interferon stimulated genes (ISGs) which establish a non-specific antiviral state within the cell [8, 30].

IFN-gamma has antiviral activity in vitro and has antiviral activity against HCV in cell culture systems but does not seem to have any effect on HCV RNA levels in humans [8].

IFN-alpha binds to cells surface receptor leading to the activation of JAK1 and Tyk2.The activated kinases leads to the activation of transcription proteins STAT 1 and STAT 2.The activated STAT 1 / 2 complex which is translocated to the cell nucleus where it combines with IFN-regulatory factor 9 (IRF-9) to form a complex that binds on the cell DNA leading to the expression of multiple ISGs. Recombinant interferon alpha which is given exogenously to patients binds to and activates cellular receptors leading to the same response cascade, that occurs with endogenous production[8, 30].

Along with its antiviral action interferon stimulates memory T-cell proliferation, prevents T-cell apoptosis, stimulates NK cell activation and dendritic cell maturation. Interferon might also prevent immune exhaustion and enhance the adaptive HCV specific immune responses[9, 30].

1.35 Mechanism of action of Ribavirin

Ribavirin is a purine nucleoside analogue and has broad spectrum antiviral activity. It was initially used as monotherapy as an antiviral aerosol in children with respiratory syncytial virus infection (RSV)in the mid 1980's before the discovery of the hepatitis C virus and for the treatment of lassa fever [176]. The mechanism of action of ribavirin can be divided into direct and indirect mechanisms.

1.36 Indirect mechanisms of action of ribavirin

Indirect mechanisms of action include enhancement of host T cell immunity against viral infections by switching the T cell phenotype from type II to type I, and inhibition of the host enzyme inosine monophosphate dehydrogenase (IMPDH). Animal models have shown an enhanced immune response of type I cytokines in lymphoid organs when induced by ribavirin [176] .Various studies have found that PBMC's of patients with chronic HCV infection showed enhanced IFN-γ expression,TNF-ά production, increased IL-2 production and decreased IL-10 expression in response to ribavirin[176].Initially when ribavirin was used to treat RSV in children it was found that not only did it eradicate the virus but also reduced airway hyperactivity which is believed to be associated with a type 2 cytokine response[176].Inhibition of IMPDH is a rate limiting step in the synthesis of guanosine triphosphate (GTP).Inhibition of GTP can lead to suppression of viral RNA replication. Studies conducted in the HCV subgenomic replicon system have shown that ribavirin, mycophenolic acid and VX-497 have shown inhibitory activity against the hepatitis C virus [177].

1.37 Direct mechanisms of action of ribavirin

Direct mechanisms of action include Inhibition of HCV-NS5b encoding RNA dependent RNA polymerase (RdRp), and acting as a direct RNA mutagen that drives a rapidly mutating RNA virus over the threshold. Ribavirin undergoes intracellular phosphorylation to ribavirin mono (RMP), ribavirin di (RDP) and ribavirin triphosphatse (RTP).Various studies have shown that the ribavirin derivative RTP weakly inhibits RdRp in all six HCV genotypes. It was also found to have weak inhibitory activity against HCV viral replication which may be correlated with its activity against HCV polymerase as ribavirin and its products are inactive against other HCV targets (e.g.NS3 protease, RNA helicase and the other ribosomal entry sites)[178]. Crotty et al has suggested that ribavirin can act as an RNA mutagen, the virus mutates and its infectivity is reduced. When using the poliovirus model to understand the mechanism of action of ribavirin,

studies have shown that in vitro use of ribavirin triphosphate (RTP) by the polio virus RNA polymerase which incorporates ribavirin and becomes mutagenic, ribavirin incorporation led to a decrease in polio virus production i.e. less than 0.00001% in cell culture .This has shown that ribavirin had decreased RNA replication and at the same time causing a significant decrease in infectivity by acting as a mutagen[178].

An interesting clinical observation is that ribavirin monotherapy had minimal effect on HCV viraemia even though the serum alanine transaminase (ALT) levels were found to be reduced in a large proportion of patients with chronic HCV infection[176].

1.38 Assessment of Liver Fibrosis

Development of hepatic fibrosis with the subsequent progression to cirrhosis is the outcome for a significant proportion of patients infected with the hepatitis C virus despite the individual variation in time between acquiring infection and progressing to cirrhosis. The gold standard to assess the degree of hepatic damage is with the help of a liver biopsy. In order to assess the stage of the liver biopsy, standardized scoring systems such as the Ishak, METAVIR and Knodell scoring systems are used to determine the amount of inflammation and scarring present in the liver. The Ishak system is a revised version of the older histological activity index and classifies grading and staging separately. Liver fibrosis is classified as *absent=0, mild=1-2 ,moderate=3-4 & severe/cirrhosis=5/6.*

Table 1.4 Different Scores to grade Liver biopsy

Stage	IASL	Metavir	Ishak
0	No fibrosis	No fibrosis	No fibrosis
1	Mild fibrosis	Periportal fibrotic expansion	Fibrous expansion of some portal areas with or without short fibrous septa
2	Moderate fibrosis	Periportal septae 1 (septum)	Fibrous expansion of most portal areas with or without short fibrous septa
3	Severe fibrosis	Porto-central septae	Fibrous expansion of most portal areas with occasional portal to portal bridging
4	Cirrhosis	Cirrhosis	Fibrous expansion of most portal areas with marked bridging (portal to portal and portal to central)
5			Marked bridging (portal to portal and portal to central) with occasional nodules (incomplete cirrhosis)
6			Cirrhosis

Table 1.4 reproduced from AASLD guidelines [140]

However due to the cost and invasive nature of a liver biopsy and the reluctance of patients to undergo the procedure, interest has grown in the development of non invasive markers to assess hepatic fibrosis progression. The most commonly used procedure is an ultrasound which helps to identify the structure of the liver i.e. irregular margins or a nodular liver which are hallmarks of cirrhosis[179].

Biochemical parameters which can be identified in the patients serum and are believed to correlate with the fibrogenic process within the liver have also been described, and are classified into two categories as described below

i)Direct Markers: They are the parameters reflecting the actual fibrosis and the extracellular matrix turnover.

ii)Indirect Markers: These are based on altered liver function tests which are routinely observed in standard clinical practice[179].

Table 1.5 : Commonly used direct and indirect markers which are used to assess fibrosis.

Direct Markers	**Indirect Markers**
Hyaluronic acid	Platelet count
Laminin	AST,ALT
Procollagen III	γ GT
Type IV collagen	γ- globulins
Metalloproteinases	Albumin
Inhibitors of metalloproteinase's	Prothrombin time

Table 1.5 reproduced from World J Gastroenterol 2009; Sebastiani, G [179]

A very popular method which is used to judge liver fibrosis is by assessment of liver stiffness with the help of transient elastography, measured by fibroscan. Various studies have suggested a cut off 7.1 kPa-8.7 kPa for significant fibrosis and for cirrhosis is defined with a cut off of > 12.5 kPa on fibroscan. [180]

It can be difficult to assess liver stiffness in obese people, those patients with mild fibrosis and additionally there is an inter-operator variability with the results. Many studies have suggested the use of a fibroscan along with a non invasive marker to assess the progression of fibrosis in patients with chronic HCV infection [179, 180].

1.39 WORK IN THIS THESIS

Studies to date have failed to identify the most effective treatment regimes for patients with chronic genotype 3 HCV infection. There is controversy regarding the role of cirrhosis in modifying response and disagreement regarding the impact of ethnicity on treatment outcome. Given the importance of genotype 3 HCV in the global epidemic and the lack of high quality research into this genotype the purpose of this work was to address some of the deficiencies in our understanding of the optimal management of this strain of hepatitis C.

We hypothesised that patients from the Indian sub-continent (South Asians) would respond differently to therapy with pegylated interferon and ribavirin than Caucasians and we hypothesised that an analysis of viral and host factors underlying the difference would reveal new insights into the virology of Genotype 3 HCV infection. We further speculated that increasing the duration of therapy in 'difficult to manage' patients with Genotype 3 HCV would improve response rates and overcome some of the negative factors. Three different research methodologies were used including a meta-analysis of factors associated with treatment failure in patients with genotype 3 HCV, virological and immunological studies on patients with genotype 3 HCV who had failed to respond to therapy and a clinical trial evaluating extended duration therapy in patients with Genotype 3 HCV infection and cirrhosis.

Chapter 2

General Methodology

2.1 Materials and Methods

2.1.1 Overview

This thesis examines the clinical, virological and immunological factors associated with treatment failure in patients with genotype 3 chronic HCV infection. Three different research methodologies were used:

A) A meta-analysis of factors associated with interferon and ribavirin treatment failure in S. Asian patients with genotype 3 HCV in comparison to Caucasian patients. This involved a retrospective review of 639 patients treated at various Liver Units throughout the UK.

B) A randomized controlled clinical trial comparing the response to treatment with interferon and ribavirin for 48 weeks compared to the standard therapy of 24 weeks in patients infected with HCV genotype 3 and who had cirrhosis or advanced fibrosis.

C) A study of virological and immunological factors associated with relapse following therapy with interferon and ribavirin in patients chronically infected with genotype 3 HCV.

2.1.2 A retrospective review of factors associated with treatment failure

An anonymised retrospective audit was performed examining patients chronically infected with genotype 3 HCV infection who had been treated with interferon and ribavirin at four UK liver units, Birmingham University Hospital, The Royal London, Bradford Royal Infirmary and St. Mary's NHS Trust. Case notes of a total of 639 patients were examined and data on factors thought to influence response to therapy.These were age and ethnicity, liver biopsy findings, cirrhosis, response to therapy, viral subtype, duration of therapy and compliance with therapy, were collected.

The patients from the Indian subcontinent i.e. Pakistan, Bangladesh and India are referred to in the dataset as South Asian while the rest of the population are considered non Asian.

Cirrhosis was defined as patients with a fibrosis score 5/6 or radiological evidence of cirrhosis. Rapid virological response (RVR) was defined in the dataset as week 4 virus not detectable by local virological testing in the hospital's laboratory, early virological response (EVR) was defined as a >2 log drop in viral load by week 12 of treatment. Patients who discontinued treatment early had their last viral load measurement carried forward and used as the end of treatment virological response. Patients who were followed up post treatment were further characterised by the treatment outcome as non responders, relapsers or as having a sustained virological response (SVR). Non-responders were defined as patients in whom the HCV RNA

was never undetectable during therapy; relapsers were defined as patients whose viral load was undetectable at the end of therapy but became HCV RNA positive during follow-up and SVR's were defined as patients whose viral load was undetectable 24 weeks after the end of therapy.

The data collected was analysed anonymously using first a univariable analysis to look at which of the factors had an impact on the response to treatment and, once those factors were identified, a multivariable analysis was completed with the help of an experienced statistician to further analyse factors which affect response to treatment.

2.1.3 Study sites:

The study was conducted in four UK treatment centres. A list of the treatment centres and the team involved is shown in Table 2.1.1:

Table 2.1.1 The Treatment Centres and the teams involved

Name of the Hospital/Trust/University	Name of the principal consultant and their main team members
Queen Marys University of London & Barts and the London NHS Trust The Royal London Hospital North East London	**Professor G.R. Foster-** Consultant Dr. Dania Shoeb – Clinical Research Fellow/PhD student/study coordinator Dr. Masaud –MSc. student Opal Greyson –CNS nurse specialist Jean Baker - CNS nurse specialist
Imperial College, University of London & St Marys Hospital NHS Trust, London	**Dr. Ashley Brown-**Consultant Dr. Henning Pflugard –Exchange student
Bradford Royal Infirmary Hospital Bradford	**Dr Sulleman Moreea-**Consultant Dr. Ruchit Sood –SpR Gastroenterology
Birmingham University Hospitals NHS Trust	**Dr David Mutimer-** Consultant Dr Dennis Freshwater – Consultant Dr. Ian Rowe –Clinical Research Fellow

Ethical approval was obtained for all 4 centres for this study.

2.1.4 Data collection and statistical analysis

A master database (Excel spreadsheet) was established and maintained by the study co-ordinator (DS).Data from each site was entered into the spreadsheet locally by review of the clinical/nursing records. Data was, in the main, entered by members of the local treating team and each site was visited by DS when data was reviewed with the local clinical team. At the completion of the study the data was cleaned and duplicates excised. Data queries were resolved by contacting the study site and incomplete entries were removed (e.g. incomplete details available-no ethnicity, patient was in the middle of treatment and hadn't attained an SVR). The data was then locked and anonymised and each patient was given a unique ID number. Statistical analysis was performed by SPSS version 16 and statistical support was provided by Caroline Sabin-statistician at the University College, London. Further statistical help was also sought from Jessica Talbott.

2.1.5 Analysis of non-invasive markers of liver fibrosis

To examine whether advanced liver fibrosis in Asian patients with genotype 3 HCV could be detected using non-invasive serological studies we completed a sub-study evaluating a number of non-invasive markers of liver fibrosis. 68 patients with Genotype 3 HCV who had undergone a liver biopsy and in whom stored serum samples taken within 6 months of the liver biopsy were available were selected from the study. Serum from these patients was analysed using the enhanced liver fibrosis test (ELF test – kindly performed by IQUR). The ELF test is a non-invasive test to assess liver fibrosis in patients with history of chronic liver disease and alcohol abuse. The ELF test combines three serum markers (e.g. Hyaluronic acid, procollagen III amino terminal peptide and Tissue inhibitor of metalloproteinase 1) which have been shown to correlate with the level of liver fibrosis assessed by liver biopsy. The algorithm measures each of the markers by immunoassays, to create an ELF score.

The same cohort of patients were used to assess other markers of liver fibrosis – specifically the APRI and FIB-4 scores as well as an assessment of cirrhosis using the AST:ALT ratio. Results from the local laboratory from a blood sample taken within 6 months of the liver biopsy were used to calculate the appropriate markers. APRI was calculated as serum AST to platelet ratio index; FIB-4 was calculated from platelet count, alanine transaminase, aspartate transaminase, and age that predicts fibrosis and cirrhosis and AST:ALT ratio was calculated using AST and ALT values from the same blood draw.

2.2 A randomized controlled clinical trial to evaluate duration of treatment with pegylated interferon (Pegasys) and ribavirin(Copegus) in patients infected with HCV Genotype 3 and who had cirrhosis or advanced fibrosis.

This trial was a multicentre, open labelled, randomised phase IV trial comparing 24 weeks therapy with 40 KD pegylated interferon alpha 2a and ribavirin 800 mg to 48 weeks therapy with 40 KD pegylated interferon alpha 2a and ribavirin 800 mg in patients with genotype 3 chronic HCV with advanced fibrosis (modified Ishak fibrosis score of 4 or more).

The study protocol was prepared by Professor Foster and myself whilst working in the department as an MSc student. I was also involved in, submitting the protocol for local ethical approval and was responsible for recruiting, monitoring and managing patients at the Royal London Hospital site. I worked alongside the study co-ordinator Carol Douglas in completing the central monitoring, answering trial related queries from the different sites, reporting adverse events (AE) and severe adverse events (SAE). I was involved in the setup and data entering onto the e-CRF. This study gained approval for initiation in 2007 and the last study patient finished their follow up visit in June 2012.

An investigators protocol was prepared and distributed to the recruiting centres a copy of which is included in Appendix 8.1.2. The sponsors of the study were Roche.

2.2.1 Trial Design

The trial was designed so that a total of 140 treatment naïve patients were randomized (1:1) at baseline to either receive 24 weeks of treatment (Group A) or 48 weeks of therapy (Group B). Both groups received 180µg pegylated interferon alpha 2a (Pegasys) weekly in combination with 800mg Ribavirin (Copegus) daily for the treatment duration. Both groups were followed up for 24 weeks post treatment.

The primary objective of the study was to compare the efficacy and safety of 180µg of pegylated interferon alpha 2a given weekly in combination with Ribavirin (800mg) daily for 24 weeks with the efficacy and safety of treating with the same dose for 48 weeks in Genotype 3 HCV infected individuals with liver cirrhosis (modified Ishak fibrosis score of equal to or greater than 4 **OR** the presence of radiological and/or endoscopic features of cirrhosis).

In addition the kinetics of the response in each group was analysed by monitoring HCV-RNA over the course of the treatment and follow-up. Also treatment outcome was evaluated in each

group relative to the viral load and the fibrosis score at the baseline and the insulin resistance, age and gender of the patient[181].

2.2. 2 Recruitment of patients:

A total of 140 treatment naïve patients were recruited at a total of 13 centres in the UK. The centres involved are listed in Table 2.2.1

Table 2.2.1 Study centres and Principal Investigators.

Centre Name	Principal investigator
Royal London	Graham Foster
Birmingham	Dennis Freshwater
St. Georges	Daniel Forton
Manchester	Javier Villar
Nottingham	Stephen Ryder
Royal Free	Geoffrey Dusheiko
Cambridge	Graeme Alexander
Imperial/St Marys	Shahid Khan
Cardiff	Andrew Freedman
Bristol	Fiona Gordon
Oxford	Jane Collier
Glasgow	Peter Mills
Bradford	Sulleman Moorea

All patients were treatment naive HCV RNA positive and anti-HCV positive chronically infected with evidence within the previous 18 months in a liver biopsy of a modified Ishak fibrosis score of equal to or greater than 4 or radiological and/or endoscopic features of cirrhosis. All of the patients were over 18 years of age, and were not co-infected with hepatitis B virus (HBV) or HIV. The patients all had compensated liver disease (Child-Pugh Grade A) and a platelet count

≥ 30,000 cells/mm^3 and neutrophil count > 600 cells/mm^3. Patients were randomised to either 24 or 48 weeks treatment at the start of the study by the study co-ordinator.

Patients were excluded from the trial if they were expected to need alternative antiviral therapy with established or perceived activity against HCV at any time during their participation in the study or had another significant cause of liver disease. Patients were also excluded if they had poorly controlled diabetes, severe retinopathy, were at risk from anaemia, a history of immunologically mediated disease, a history of severe cardiac disease, a history of severe psychiatric disease or were pregnant. All of the patients were screened for possible health difficulties prior to being accepted on to the trial.

Other medication during the course of treatment was monitored and strict guidelines, outlined in the investigator's protocol (Appendix 8.1.2), were followed. In addition patients were discouraged from alcohol consumption and were asked to consume no more than an average of 20g alcohol daily[181].

2.2.3 Clinical and laboratory assessments during the trial.

The patients treated for 24 weeks were monitored clinically at weeks 4, 12 and 24 during therapy and at weeks 36 and 48 post therapy. Patients treated for 48 weeks were monitored clinically at weeks 4, 12, 24, 36 and 48 during therapy and at 72 weeks post therapy. Blood samples were taken at each monitoring visit to assess the patient's clinical health and to monitor HCV RNA levels.

Patients who had severe or clinically significant reductions in haemoglobin or platelet/neutrophil counts had either the dose of ribavirin or the dose of pegylated interferon reduced. All dose adjustments were recorded. All patients who discontinued treatment prematurely were assessed for safety at 4 weeks and again at 12 weeks post their last dose of study medication. Patients who discontinued from treatment prematurely and whose HCV RNA was undetectable at last HCV RNA assessment (4 weeks post last dose or other) were assessed at 12 weeks and 24 weeks post their last dose of study medication.

Any clinical adverse event, serious adverse event or abnormal laboratory test value occurring during the course of the study, irrespective of the treatment received by the patient, was reported to the sponsor within 1 working day of occurrence. The full requirements of the ICH Guideline

for Clinical Safety Data Management, Definitions and Standards for Expedited Reporting, Topic E2 were be adhered to (see Appendix 7.1.2)

30 patients were treated at the Royal London. These patients were monitored and had blood taken more frequently as outlined in section 2.3[181].

2.2.4 HCV RNA measurement

The study was initiated with a single, central viral laboratory (IQur) performing the HCV RNA analyses using a commercial viral load assay (Roche TaqMan). During the course of the study the contract for the testing was transferred to Lab21 who were unable to comply with the study testing requirements. The contract was therefore transferred to The Royal London Hospital Virology Department who performed the viral load tests whilst arrangements were made to transfer the contract to a third testing laboratory (The Doctors Laboratory, London). All accredited laboratories used a standardised Roche Taqman assay with a lower limit of detection of <30 IU/ml. During the transfer of the virology testing between the different laboratories a number of samples were tested by local laboratories using the Roche Taqman assay with a lower limit of detection of <15 IU/ml.Where possible samples were re-tested in the central laboratory and, in the event of any discrepancy, it was agreed that the central laboratory testing would be used.

2.2.5 Statistical model and sample size

Audit data from participating centres suggested that sustained virological response rates of approximately 50% may be expected in this population with difficult to treat genotype 3 chronic HCV. To justify the additional expense and side effects of therapy an increase in sustained virological response rates to 75% were required. Based on standard statistical criteria a total of 60 patients per treatment arm were required to provide a >80% chance of detecting a significant difference. We assumed that 10% of patients would withdraw from the study so a total of 66 patients per treatment arm were required so we recruited 70 patients per arm.

2.2.6 Analysis populations

The four populations analyzed in this study were:

a) All patients randomised according to the treatment group to which they were assigned at randomisation regardless of the treatment they actually received after randomisation.

b) All patients randomized who received at least one dose of either study medication after screening. This population is referred to as the intent-to-treat (ITT) population.

c) The per-protocol population excluded a randomised patient if the patient met any of the exclusion criteria. Analyses using the per-protocol population were to be performed only if >10% of patients were excluded from the ITT population.

d) The safety population included only patients who received at least one dose of either study medication and had at least one post baseline safety assessment. Patients were analysed according to the treatment they actually received, regardless of whether they discontinued prior to week 20.

All primary and secondary efficacy endpoints were analyzed using the ITT population[181].

2.3 Virological and immunological factors associated with relapse

To examine the virological and immunological factors associated with relapse we further studied the 30 patients who were enrolled in the trial (described in section 2.2) at The Royal London who had chronic HCV genotype 3 infections and followed up post treatment more extensively. These patients had completed a course of combination treatment with pegylated interferon and ribavirin and blood samples were taken from the end of treatment weekly for six consecutive weeks. The first 10 patients were bled weekly to look at viral relapse and viral diversity post relapse. Early data from other experiments in the laboratory on 4 patients had suggested that changes in T regulatory cells may play a significant role in predicting response to treatment. This observation led to the hypothesis that changes in T regulatory cells may predict or be associated with relapse so this study was extended to test this hypothesis further.

The FACS analysis used in this study was done with the help of a senior immunologist Dr. L Hibbert.

The T cell analysis was replicated in a group of healthy controls.

All of the studies had ethical approval.

2.3.1 Materials and Antibodies

A) Antibodies

The majority of the directly labelled antibodies and their recommended isotype controls were obtained from BD Biosciences, Oxford,UK. Rat anti-FoxP3 Pacific Blue and rat anti-IL17a PE were obtained from eBiosciences. The volumes of antibody used were those recommended by the manufacturer. The antibodies used and the combinations they were used in are listed in Table 2.3.1

B) Unless stated otherwise all tissue culture and staining reagents were obtained from Sigma and Invitrogen.

C) Complete medium

RPMI-1640(PAA Labs Ltd) with 10% heat-inactivated foetal calf serum, 2mM glutamine and 100units/ml penicillin and 0.1mg/ml streptomycin (PAA Labs Ltd)

D) 4% paraformaldehyde.

Paraformaldehyde dissolved in PBS and used within 1 week of making.

E) FACS buffer.

PBS with 2% foetal calf serum, 1.23mM EDTA and 0.02% sodium azide

Table 2.3.1. Staining of PBMC's for FACS analysis

Tube 1		**Tube 2**		**Tube 3**		**Tube 4**	
antibodies	µl ab/100µl	Isotype controls	µl ab/100µl	antibodies	µl ab/100µl	Isotype controls	µl ab/100µl
CD3 PerCP-Cy™5.5	5.0	Mu IgG_1 PerCP-Cy™ 5.5	2.4	CD3 PerCP-Cy™5.5	5.0	Mu IgG_1 PerCP-Cy™ 5.5Pe5.5	2.4
CD4 PE-Cy™7	5.0	Mu IgG_1 PE-Cy™7	1.2	CD4 PE-Cy™7	5.0	Mu IgG_1 PE-Cy™7	1.2
CD25-PE	20.0	Mu IgG_1 PE	5.0	CD8.0-Pacific Blue	1.0	Mu IgG_1 Pacific Blue	1.0
CD19 APC	5.0	MuIgG_1 APC	2.5	CD69-PE	20.0	Mu IgG_1 PE	5.0
CD8-Pacific Blue	1.0	Mu IgG_1 Pacific Blue	1.0	CD56 APC	5.0	Mu IgG_1 APC	5.0

Tube 5A		**Tube 5B**	
antibodies	µl ab/100µl	Isotype controls	µl ab/100µl
CD3 PerCP-Cy™5.5	5.0	Mu IgG_1 PerCP-Cy™ 5.5	2.4
CD4 PE-Cy™7	5.0	Mu IgG_1 PE-Cy™7	1.2
CD45RO APC	20.0	Mu IgG_{2A} APC	2.5
CD62L-PE	5.0	Mu IgG_1PE	5.0
CD8-Pacific Blue	1.0	Mu IgG_1 Pacific Blue	1.0
CD27-FITC	20.0	Mu IgG_1 FITC	5.0

Table 2.3.1. Staining of PBMC's for FACS analysis (Continue…)

Tube 6A		Tube 6B	
antibodies	µl ab/100µl	Isotype controls	µl ab/100µl
CD3 PerCP-Cy™5.5	5.0	Mu IgG_1 PerCP-Cy™ 5.5	2.4
CD4 PE-Cy™7	5.0	Mu IgG_1 PE-Cy™7	1.2
CD27-FITC	20.0	Mu IgG_1FITC	5.0
Cd25-APC	5.0	Mu IgG_1APC	2.5

After washing away the fixative and permeabilizing the cells

IL17A-PE	2.0	Rat IgG_{2a} PE	1.0
FoxP3-Pacific Blue	2.0	Rat IgG_{2a} Pacific Blue	1.0

2.3.2 Sampling schedule and blood processing

30 patients from the STEPS trial being treated at the Royal London were bled weekly from the end of treatment for six weeks post treatment and monitored for virological relapse. The first 10 patients had 9mls of blood taken first thing in the morning, 4mls into a plain tube for serum sampling and 5ml in an EDTA tube for viral titre analysis. The next 20 patients had an additional 30mls of blood taken into Li Heparin tubes for separation of peripheral blood mononuclear cells (PBMC) for FACS analysis and storage.

2.3.3 Flow Cytometry Analysis

PBMC were isolated from 10ml of heparinised blood by centrifugation over 10ml Ficoll (Ficoll-paque plus, GE Healthcare) at 2500 rpm for 20 minutes. Cells were taken from the interface and washed twice in RPMI then resuspended in complete medium.

The cells were divided into 3 tubes. One tube of cells was fixed for a minimum of 30 minutes with 4% paraformaldehyde in PBS, washed once with FACS buffer, resuspended in 400µl of buffer and separated into tubes 1-4 for staining. The cells were stained with directly labelled

antibodies or isotype controls as shown in Table 2.3.1, on ice and in the dark for 30 minutes. The cells were then washed in FACS buffer and resuspended in 300µl of the same buffer for analysis.

A second tube of cells was divided in half, tubes 5A and 5B, and the cells were washed with FACS buffer, resuspended in 100µl of buffer and stained at 4^0C in the dark for 30 minutes, with antibodies to surface markers (Tube 5A) or the isotype controls (Tube 5B) as shown in Table 2.3.1. The cells were washed once and fixed with 4% paraformaldehyde. Prior to analysis the cells were washed with FACS buffer once more and resuspended in 300µl FACS buffer.

The third Tube 6A) or isotype controls (Tube 6B) as shown in Table 2.3.1 for 30mins at 4˚C in the dark. Cells were washed once and fixed overnight in 500µl fixative from the Intracellular Fixation and Permeabilization kit (e Biosciences). On the following day the cells were washed twice in permeabilization buffer, resuspended in 100µl of the buffer and stained with anti-IL17 and anti-FoxP3 (Tube 6A) or isotype controls (Tube 6B) as listed in Table 1 for at least 30 min on ice and in the dark. The cells were then washed twice in permeabilization buffer before resuspending in FACS buffer for FACS analysis.

FACS analysis was performed using the LSRII FACS machine and analysed using BD DIVA software (Becton Dickinson). The compensation was set up using BD compensation beads by Dr Linda Hibbert and Dr Gary Warnes.

2.3.4 Virological Analysis; plasma samples

Pre-treatment samples were taken and stored at -70 C. HCV RNA in plasma was monitored throughout treatment and once treatment was completed patients were monitored for HCV RNA weekly and plasma samples were stored at -80^0C.

2.3.5 Analysis of HCV quasispecies

HCV RNA was extracted from the pre and the first HCV-RNA positive post-treatment samples from patients who relapsed post completion of therapy. The viral RNA from 500µl of plasma was prepared by concentrating the virus (18,500g for 70 min, 4^0C) removing 360µls of the supernatant plasma and resuspending in the remaining 140µl plasma. HCV RNA was then extracted using the QIAamp viral RNA mini kit (QIAGEN Ltd.) following the manufacturers instructions. Briefly, 560µl buffer AVL with carrier RNA was added to the HCV RNA mixed

and incubated at room temperature for 10 minutes. 560µl of ethanol was then added and the mixture transferred to the kit spin-tubes and centrifuged at 8000 rpm for 1 minute until all the sample had been load onto the column. The RNA on the columns was washed sequentially with buffer AW1 and AW2 then eluted into a clean eppendorf with 60µl AVE elution buffer and 2µl RNAsin (40U/µl Promega) added. The HCV RNA was then stored in 3 aliquots at -80^{0}C.

2.5µl RNA was reverse transcribed using SuperScript^{TM} III reverse transcriptase (Invitrogen) and the primer F4 Rev at 55^{0}C for 55 min and 70^{0}C for 15 min, and treated with 10U RNAse H (Ambion) for 20minutes 37^{0}C. Nested PCR was performed on 2.5µl of the cDNA using the primers F4 Rev and UTR 277-For followed by a second round of PCR with 2.5µl of substrate using primers 745-For and 2982-Rev (Table 2.3.2 and ref 1) using High Fidelity *Taq* DNA polymerase (Roche). The cycling conditions for the 1st round PCR were 94^{0}C for 2 min followed by 40 rounds of 94^{0}C for 15s, 58^{0}C for 30s, 68^{0}C for 4 min with a final incubation at 68^{0}C for 10 min. The 2nd round PCR conditions were 94^{0}C for 2 min followed by 30 rounds of 94^{0}C for 15s, 50^{0}C for 30s, 72^{0}C for 2 min with a final incubation at 72^{0}C for 7 min.

To obtain sequence from both the major and the minor quasispecies in the serum end-point the cDNA was serially diluted until the number of PCR positive samples was 2 in 4, this dilution was then used to produce PCR product to sequence. The cDNA of 2 of the patients only resulted in PCR product when undiluted so these PCR products were cloned using Zero Blunt^R TOPO^R PCR cloning kit (Invitrogen) according to the manufacturer's instructions. The quasispecies of 1 patient were analysed using both methods, both of which produced the same groups of quasispecies. For each of the patients sequenced 10-20 separate clonal sequences from both the pre- and first post-treatment samples were obtained.

Samples were sequenced in both directions using the Sanger sequencing service (Source Bioscience) and the primers listed in Table 2.3.2. The sequences were analysed and assembled using Vector NTI advance^{TM} 11 software (Invitrogen). The guide trees were constructed using the AlignX program within Vector NTI which uses a modified Clustal W algorithm and multiple pairwise alignments.

All PCR was carried out following the advice of Kwok and Higuchi (1989) to avoid contamination. All of the sequences of the quasispecies from the patients were compared as above and they all segregated by patient, confirming that there was no contamination between samples[182].

Table 2.3.2 Primers used for PCR and sequencing

Primer	Sequence (5'-3')	Binding Site(nt)	Source
F4 Rev	CTGGGTAGCCGTAGAAAGCACCT	3520	[183]
UTR-277	CCTTGTGGTACTGCCTGATAG	279	[183]
2982-Rev	ATAAAGCAGGCTTGTTAG	2967	[183]
745-For	TACATCCCGCTCGTCGGC	747	[183]
E2-For	GGCAACTGGGCCAAGGTCGC	1437	[183]
HVR1outerFor	ATGGCATGGGATATGAT	1293	[184]
HVR1outerRev	AAGGCCGTCCTGTTGA	1606	[184]
HVR1innerFor	GCTTGGGATATGATGATGAA	1296	[184]
HVR1innerRev	GTCCTGTTGATGTGCCA	1599	[184]
MK Rev	ACCAAGCGTACGCCCGTTGG	2753	In house

nt, nucleotide (numbering relative to the reference sequence AF009606 [185])

Chapter 3

A retrospective review of factors associated with Treatment failure in patients with Genotype 3 HCV.

3.1 Introduction

Pegylated Interferon (alpha 2a or 2b) plus ribavirin is the standard of care for patients with chronic HCV infection. The aim of treatment for chronic HCV infection is to attain a sustained virological response (SVR) which is defined as serum HCV RNA levels that are undetectable with a sensitive assay 24 weeks after cessation of treatment. This end point is associated with regression of fibrosis, decreased incidence of hepatocellular carcinoma and overall a reduced morbidity and mortality. Response to therapy is determined by the viral genotype, pivotal clinical studies show that patients with genotype 1 HCV have SVR rates of approximately 40-50%[186] whereas patients infected with genotypes 2 and 3 have SVR rates approaching 80%.

The viral kinetics of interferon alpha based regimes is complex. For patients infected with genotype 1 there is a triphasic response with an initial rapid decline followed by a plateau for 2-5 days and then a slow decline over several weeks. The triphasic decline consists of a first phase (1-2 days) with rapid virus load decline, followed by a "shoulder phase" (4-28 days) in which virus load decreases slowly or remains constant, and a third phase of a further decrease of viral load[187] . In patients with genotype 2 or 3 HCV the response is typically bimodal with a rapid first phase and a slower second phase response. Non responders to treatment can have no decline in the first phase or a decline in the first phase followed by a little or no second phase fall in HCV RNA. The initial rapid phase of decline of HCV RNA is thought to correspond to the blocking of viral replication in infected cells by Interferon alpha and efficacy of treatment depends not only on treatment regimen but also on many other factors e.g. HCV genotype, initial viral load etc. The second phase of decline occurs in patients who respond to therapy and is thought to reflect the clearance and loss of infected cells. The overall pattern of viral response to Interferon based therapy can be used to determine the chance of success of treatment and often helps to guide treatment duration in patients with chronic HCV infection.

Patients who fail to achieve an early virological response (EVR) which is defined as either an undetectable level of HCV RNA or a drop of HCV RNA level of at least 2 $\log_{10}$ IU/ml after 12 weeks of therapy, are considered highly unlikely to have an SVR. So genotype 1 patients who do not achieve an EVR are advised to stop treatment after 12 weeks but there is no stopping rule for

genotypes 2 or 3 because primary failure is very rare [134, 135, 188]. Rapid virological response (RVR) is defined as an undetectable level of HCV RNA (<50 IU/ml) at 4 weeks of treatment and is associated with a very good response to therapy in all patients [137]. Recent studies have suggested that patients who achieve an RVR may be candidates for short duration therapy and patients with genotype 1 HCV patients who achieve a RVR are advised to stop therapy after 6 months (i.e. after half of the normal duration of therapy)[189].

Various clinical trials have looked at factors affecting SVR in HCV genotype 3 patients and have determined whether or not short treatment durations are possible in patients with a RVR.

Zeuzem et al; Gastroenterology 2005 looked at whether shorter duration of treatment i.e. 16 or 24 weeks with peginterferon and ribavirin is beneficial and found that patients who were genotype 3 and had an initial high viral load may need longer duration of treatment as compared to HCV genotype 3 patients with an initial low viral load who respond well to 16 weeks of treatment with Pegylated Interferon and Ribavirin[135]. However ACCELERATE, the largest study ever conducted in HCV genotype 2/3 patients, demonstrated that 16 weeks of treatment is inferior to the current standard of care, 24 weeks of treatment and a significantly higher SVR rate was observed with 24 weeks of therapy as compared with 16 weeks of treatment.The ACCELERATE data (which involved a properly powered study) suggests that earlier studies in genotype 3 HCV showing differences in SVR rates between 12 and 24 weeks therapy may have led to erroneous results because of a small sample size (these trials are discussed in more detail in The Introduction). Other factors which may influence response to treatment may be the presence of hepatic steatosis. Zeuzem et al [136]prospectively looked at 182 patients who had chronic HCV genotype 3 infection and found an SVR of 79% but hepatic steatosis and baseline high viraemia were found to be important predictors of SVR and it was seen that patients who had hepatic steatosis had a decreased response rate. A number of studies in patients with genotype 1 HCV have suggested that host genotype may influence response,several American studies showed that African Americans have an inferior response rate to treatment compared to Caucasians[189] . For genotype 3 HCV Freshwater et al [169]showed that the Asian community had an inferior response to combination antiviral therapy when compared to non Asians suggesting that host genetic factors and race may play a role in genotype 3 HCV. An overview of the factors associated with a failure to achieve an SVR in univariable analysis were age,

advanced liver disease stage, higher serum aspartate transaminase, bilirubin, alkaline phosphatase, diabetes ,as well as lower white cell count haemoglobin and platelet count. On multivariable analysis cirrhosis, diabetes and age achieved statistical significance. However it is unclear whether host ethnicity or factors associated with ethnicity (such as diabetes, cirrhosis) are responsible for the observed reduction in SVR rates in patients from S.Asian communities[169].

Data to date on factors that predict the response to therapy in patients with genotype 3 HCV has relied on small scale studies and no studies have looked at a large population of genotype 3 patients. The aims of this study were to complete a meta-analysis of factors associated with treatment failure in patients with genotype 3 HCV examining the hypothesis that patients from South Asia respond less well to therapy than Caucasian patients. This involved a retrospective review of over 600 patients treated at various Liver Units throughout the UK.

Results

A total of 639 patients notes were reviewed and analysed from the four UK treatment centres i.e. Royal London Hospital, Birmingham University Hospital, Bradford Royal Infirmary, St. Mary's NHS trust (see Materials and Methods). S. Asian's were referred to as people originating from the Indian subcontinent i.e. Pakistan , India and Bangladesh.

The demography and response to treatment of the patients studied is shown in Table 3.1 and Figure 3.1.

Table 3.1: Demography of the Chronic HCV genotype 3 patients

	South Asian	Non Asian
Male	191	227
Female	126	95
Total	317	322

Figure 3.1:% SVR in genotype 3 chronic HCV patients

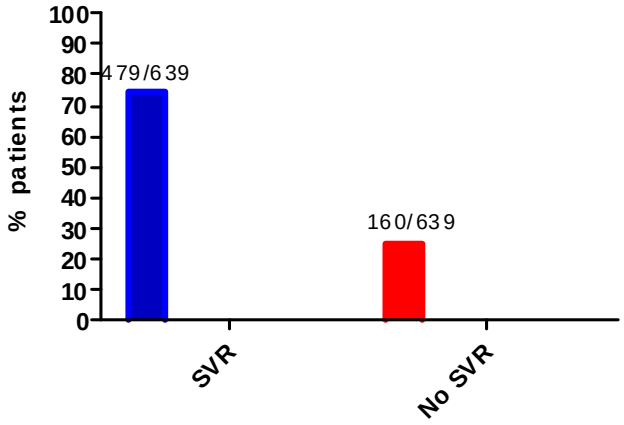
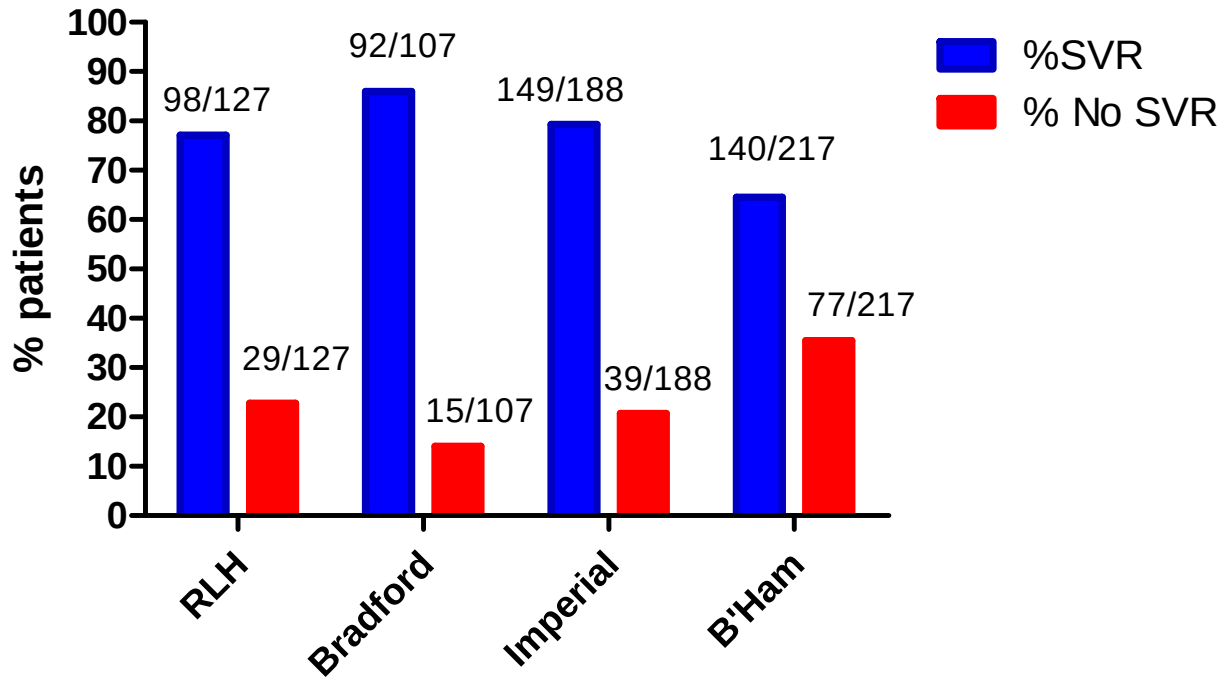


Figure 3.1 summarises the treatment outcome from the study -the overall response rate was 74.96% in our genotype 3 patient population. This is similar to the findings from the pivotal clinical studies. Figure 3.2 outlines the outcomes from the four treatment centres.

In the Royal London site a response rate of 77.16% (n=98/127) was seen, in the Bradford site a response rate of 85.98% (n=92/107) was observed, at the Imperial site the response rate was 79.25% (n=149/188) and in the Birmingham University hospital site a response rate of 64.51% (n=140/217) was seen. By univariate analysis there was no significant difference in response rates between any of the treatment centres p=0.1509 (Chi-square) (see later for details of multivariable analyses of this data set).

Figure 3.2: Comparison of the response to treatment -different centres



In view of previous studies from the Birmingham group showing differences in response to therapy in people of South Asian ethnicity we examined the impact of ethnicity on response and Figure 3.3 shows the results. Note that some data from the previous Birmingham study was included in this analysis – 95 S. Asian patients and 125 non-Asian patients from the Freshwater study were included. Of the 639 patients in our data base 317 were from S. Asia and 236 achieved an SVR 74.44% of the 322 Non Asian patients 75.46% (243/322) achieved an SVR. The difference was not statistically significant – p = 1.000 (Chi square).

Figure 3.3: Impact of ethnicity on response to therapy

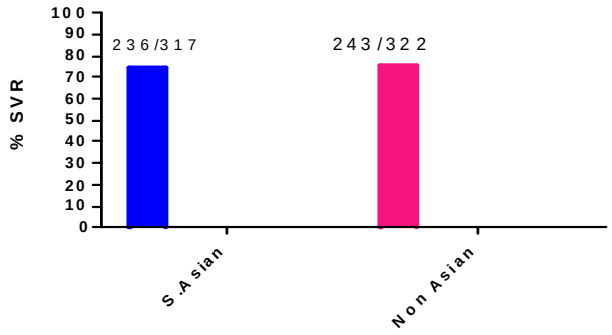


Figure 3.4: Treatment outcome by sex

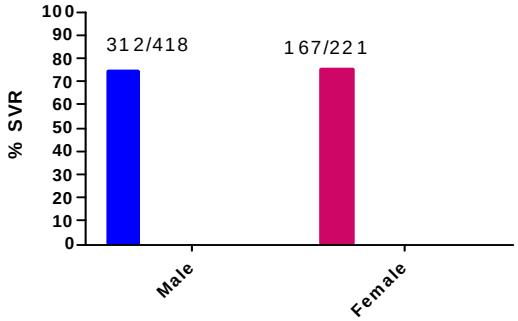
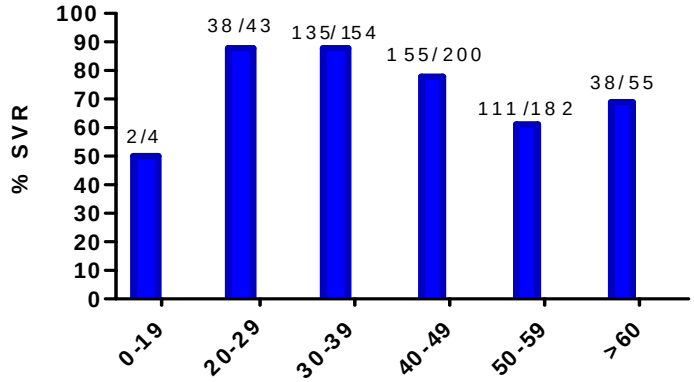


Figure 3.4 shows the impact of sex on treatment outcome - the response rate in males was 74.64% and did not differ significantly from that seen in females -75.56%. p = 0.8480 (Chi square) across different sites.

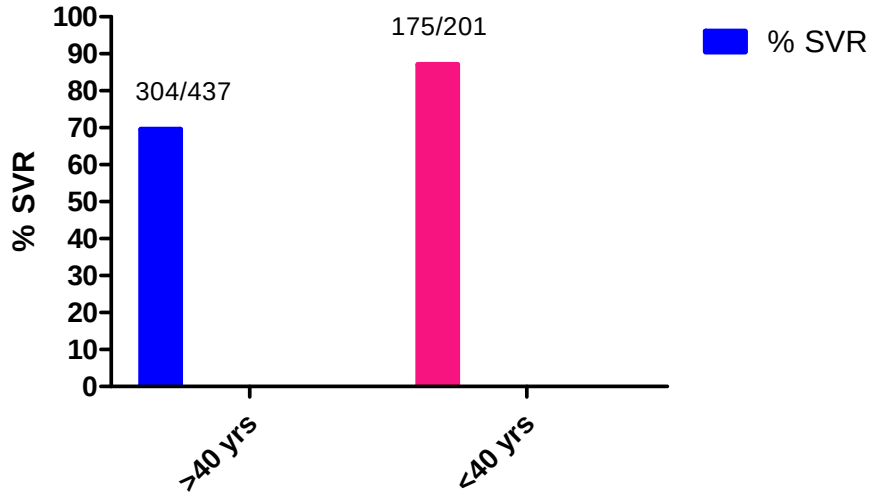
Figure 3.5: Effect of Different age groups on SVR



To examine the impact of age in more detail we stratified patient age into decades and figure 3.6 summaries the results. A response rate of 50% (n=2/4) was seen in the 0-19 years age group but the numbers in this age range were too small for meaningful analysis.

However as the treated patients age increased there was a decline in the virological response which fell from 88.4% in patients aged between 20-29 years to 69% in patients older than 60.

Figure 3.6: Effect of Age on SVR



Various studies have shown that older patients respond less well to treatment. We therefore analysed our data by patient age and divided the data into patients >40 yrs old and patients <40 years old .We initially analysed data to identify where any age related drop in response rates occurred and as illustrated Figure 3.5 we noted that responses rates in patients approaching 40 years of age had a reduced response rates.

Accordingly we analysed the dataset into >40 years of age and <40 years of age – Figure 3.6 summarises the analysis - of the 437 patients who were older than 40 years of age 69.56% (304/437) achieved an SVR. Of the 201 patients less than 40 years of age, 87.06% (175/201) achieved an SVR. This difference was found to be statistically significant p=<0.0001(Chi square).

Figure 3.7: Effect of Different age groups and ethnicity on SVR

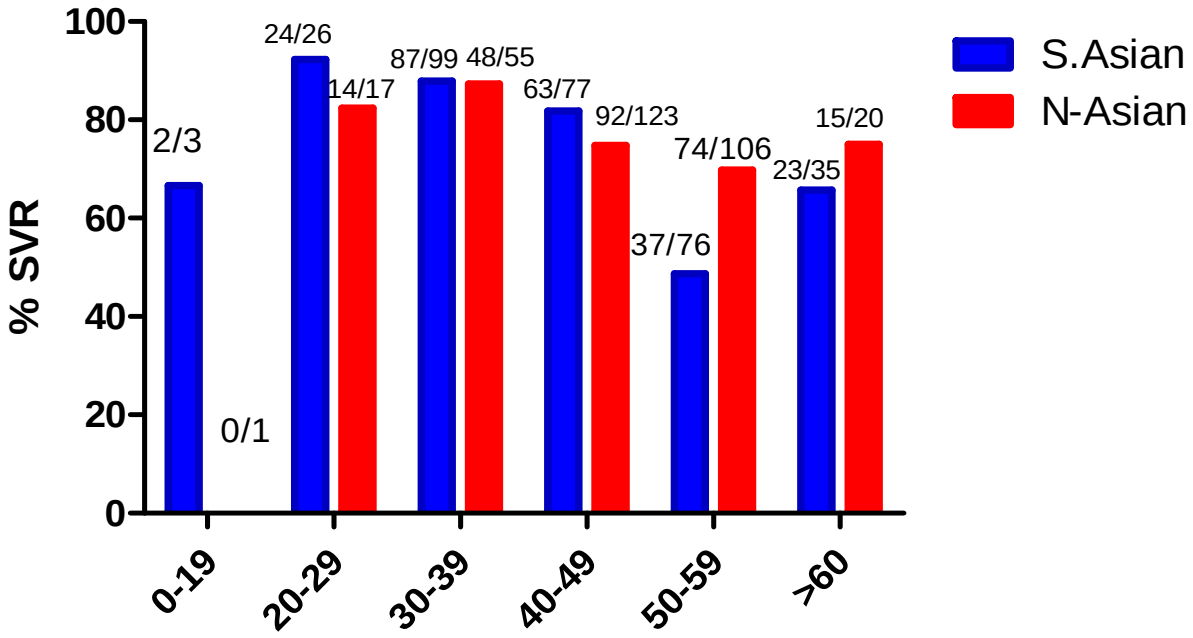
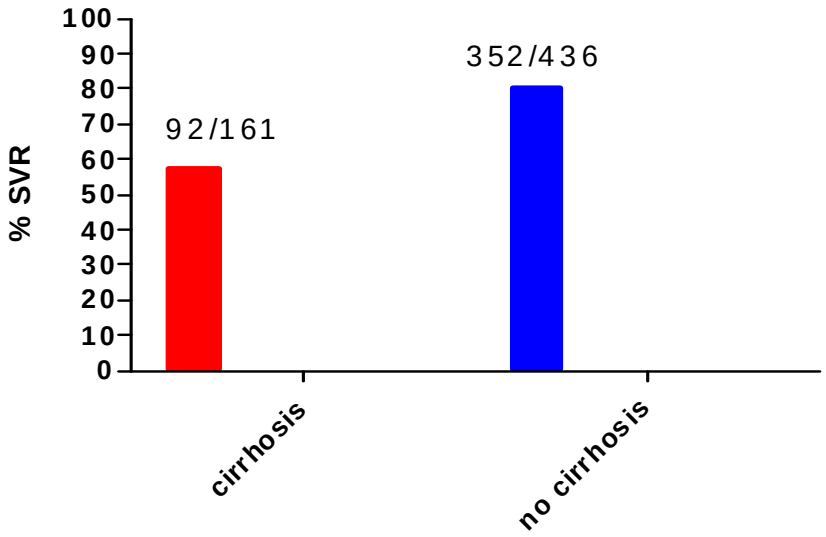


Figure 3.7 looks at the effect of different age groups and ethnicity on SVR .The dataset was stratified into S.Asian and Non Asian and their response to treatment.

It was seen that the response to treatment decreased with age >50 years in the S.Asian patients when compared to the Non Asian patients however the number of patients in the different age ranges was too small for a robust analysis

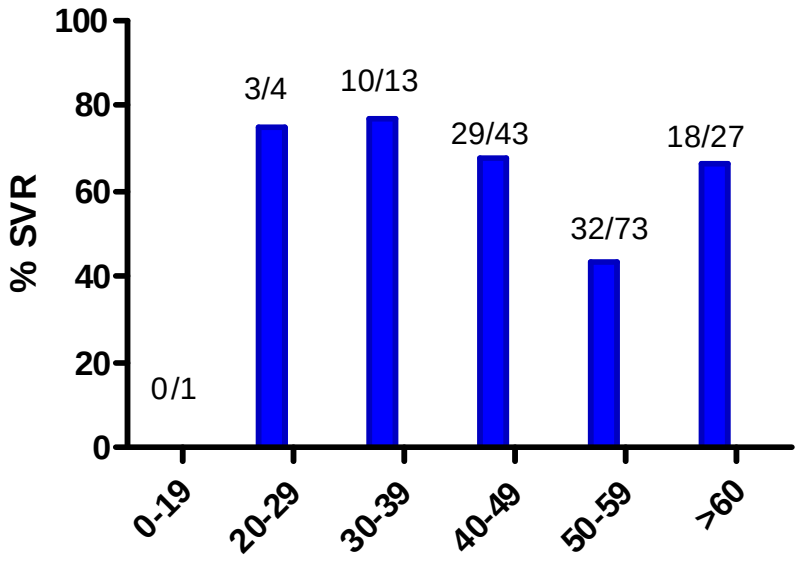
Figure 3.8: Effect of Cirrhosis on SVR



A number of studies have shown that cirrhosis decreases the response to treatment. Figure 3.8 illustrates the impact of cirrhosis on response to treatment in our cohort. (Cirrhosis was defined as a fibrosis score of 5 or 6 on liver biopsy or ultrasound evidence of cirrhosis).

We found that of the 161 patients who had cirrhosis 57.14% (92/161) had a response to treatment whilst response to treatment was seen in 80.73% (352/436) of patients without cirrhosis. This was statistically significant (p=0.0007) by univariate analysis.

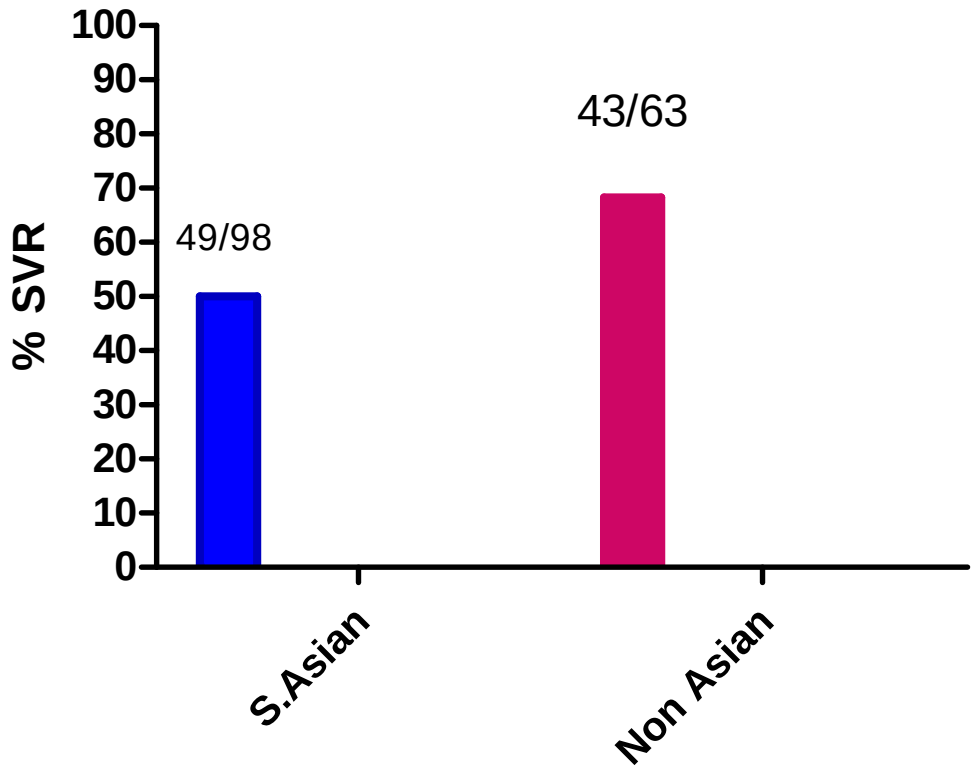
Figure 3.9: Effect of Age and Cirrhosis on SVR



In an attempt to determine whether the effect of cirrhosis was influenced by age we completed a preliminary analysis of the effects of cirrhosis at different patient ages. Figure 3.9 illustrates the result. In patients younger than 40 the SVR in patients with cirrhosis was 72% which was not significantly different from the response seen in patients younger than 40 (88.23%) who did not have cirrhosis p=0.0702 (Chi Square).

In elderly patients (>40) SVR was 55% in those with cirrhosis compared to 76.22% in those without cirrhosis p=<0.0001 (Chi Square). These data suggest that cirrhosis reduces response rate but the effect is more pronounced in the elderly cirrhotic patients.

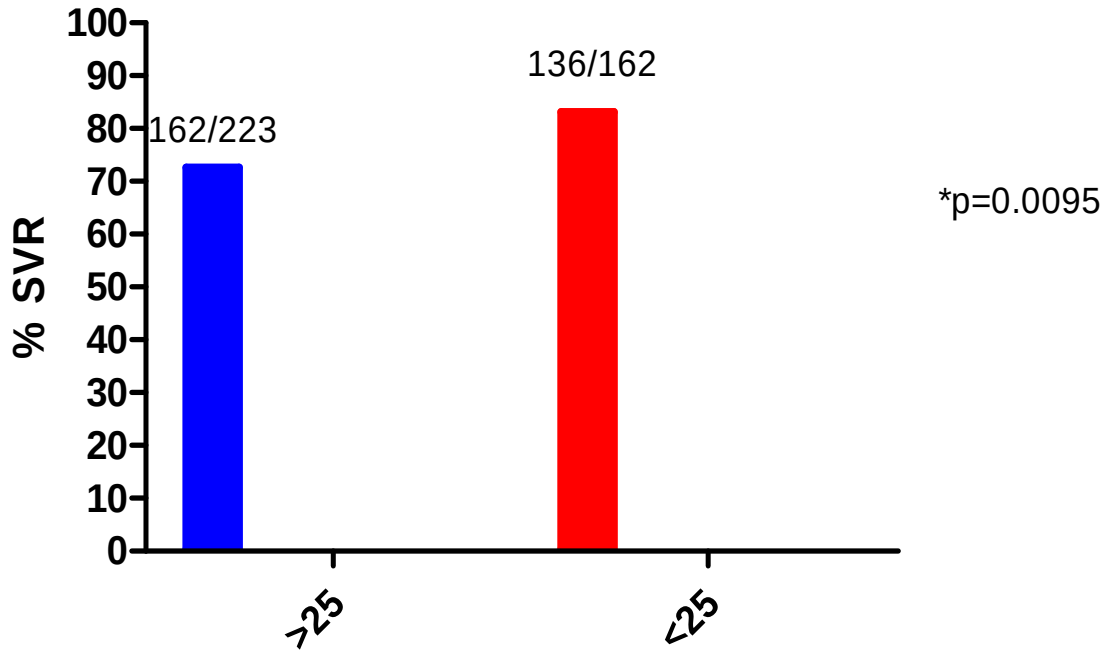
Figure 3.10: Effect of ethnicity and cirrhosis on SVR



To examine the effects of cirrhosis and ethnicity on response we examined response rates in S. Asian and non-Asian patients with cirrhosis.

Figure 3.10 shows the results – there was a significant reduction in response (50%, 49/98) in the S. Asian patients with cirrhosis compared to Caucasians with cirrhosis (68.25%, n=43/63). This was statistically significant by uncorrected, univariate analysis p=0.0237 (Chi square).

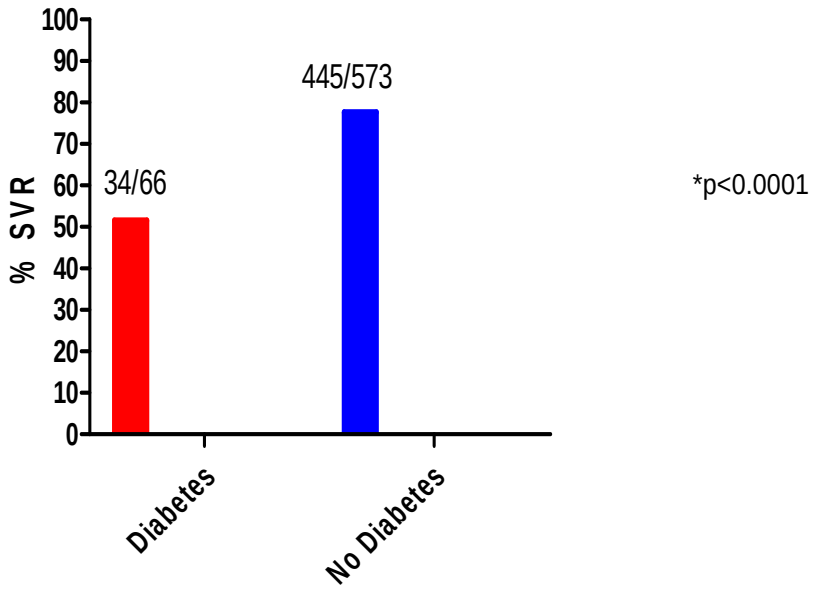
Figure 3.11: Effect of BMI on SVR



The impact of BMI on response rate was examined and Figure 3.11 shows the results. We chose a BMI of 25 as the cut off in our analysis as a recent paper suggests that people from Asia who have a BMI equal to or above 25 should be classified as overweight/obese[190] .

The 385 patients with information regarding BMI were split into > 25 BMI or < 25 BMI. The SVR seen in patients with a BMI of > 25 was 72.64% (162/223) .An SVR of 83.95% (136/162) was seen in those with a BMI of < 25. This difference was statistically significant *p=< 0.0095 (Chi-square).

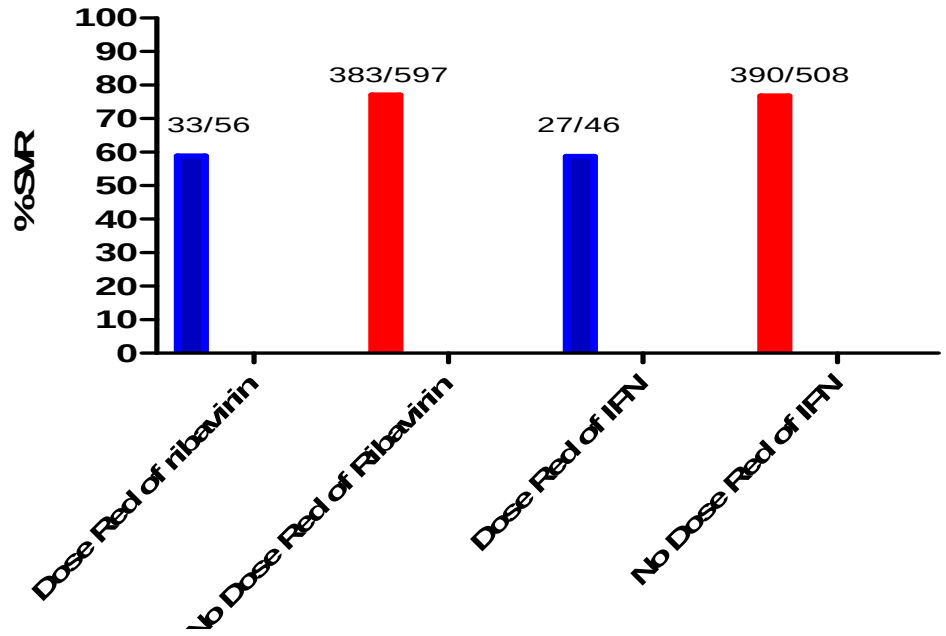
Figure 3.12: Effect of Diabetes on SVR



Many studies have shown that insulin resistance decreases the response to treatment in patients with chronic HCV infection.

We therefore examined the impact of diabetes on response and Figure 3.12 shows the results - of the 66 patients who had diabetes 51.51% (n =34/66) achieved an SVR while of those patients who did not have diabetes 77.66% (n=445/573) achieved an SVR. This difference was statistically significant p=<0.0001 (Chi square).

Figure 3.13: Effect of Dose Reductions on SVR



Previous studies, predominantly in patients with genotype 1 HCV, have shown a decrease in SVR in patients who had a dose reduction in ribavirin and/or interferon. Previous studies have defined a dose reduction of >80% as having an impact and we therefore examined the impact of a dose reduction of this magnitude. The number of patients with a dose reduction was small – 33 patients had a dose reduction of ribavirin alone, 27 patients had a dose reduction of IFN and 13 patients had a dose reduction of both drugs. Figure 3.13 shows the impact of dose reduction on treatment response rates. In the 56 patients who had a dose reduction of ribavirin, 33 (58.9%) achieved an SVR compared to 383 of 497 (77%) patients who did not have a dose reduction. This was statistically significant - p=<0.0037 (Chi Square).

A total of 46 patients had a dose reduction of interferon and 27 (58.7%) achieved an SVR whereas 76.77% (n=390/508) of patients who didn't have a dose reduction of interferon achieved

an SVR. This was statistically significant p=<0.0136 (Chi-Square). Table 3.2 describes the effect of cirrhosis, older age and South Asian ethnicity on response rates to treatment in patients with and without dose reductions of ribavirin.

Table 3.2: Effect of cirrhosis, older age and South Asian ethnicity on response rates

Variable	Proportion of patients with dose reduction of ribavirin	Response rates in patients with dose reduction	Response rates in patients without dose reduction
Cirrhosis	15.52%(n=25/161)	52%(n=13/25)	58.53%(n=72/123)
Age >40 years	10.98%(n=48/437)	56.25%(n=27/48)	71.18%(n=229/322)
S. Asian	9.46%(n=30/317)	50%(n=15/30)	77.39%(n=202/261)

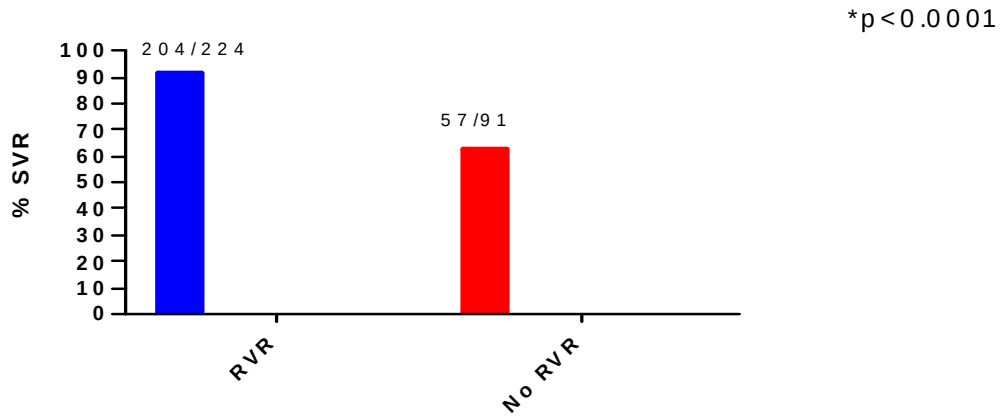
It was seen that in the 161 patients who had cirrhosis 25 had dose reduction of ribavirin and of those 52%(n=13/25) achieved an SVR whereas those cirrhotics who did not have dose reduction had a SVR 58.53%(n=72/123).

Of the 437 patients who were >40 years of age 10.98%(n=48/437) had a dose reduction of ribavirin and of those 56.25%(n=27/48) attained an SVR. Of the 322 patients who did not have a dose reduction of ribavirin 71.18% (n=229/322) achieved a SVR.

When looking at the effect of S.Asian ethnicity and its impact on dose reductions it was found that of the 30 S. Asian patients who had dose reduction of Ribavirin 50%(n=15/30) achieved a SVR as compared to 77.39%(n=202/261) who didn't have a dose reduction of ribavirin. Although the number of patients studied was small these data suggest that for 'vulnerable' patients who are at risk of treatment failure dose reductions may be associated with treatment

failure but for patients who have a high probability of response dose reductions may be less problematic.

Figure 3.14: Impact of Rapid virological response - RVR



Various studies have suggested that there is a high probability of attaining an SVR in patients who achieve an RVR – defined as undetectable HCV RNA at week 4 of treatment. Of the 178 patients who achieved an RVR 83.60% (204/244) achieved an SVR while 62.63% (57/91) of the patients who did not have an RVR achieved an SVR. This was statistically significant p=<0.0001 (Chi Square) and the data is plotted in Figure 3.14

To determine the influence of ethnicity on RVR and outcome we examined RVR and SVR rates in Caucasians and S. Asians.

Figure 3.15: Impact of Ethnicity and Rapid Virological Response

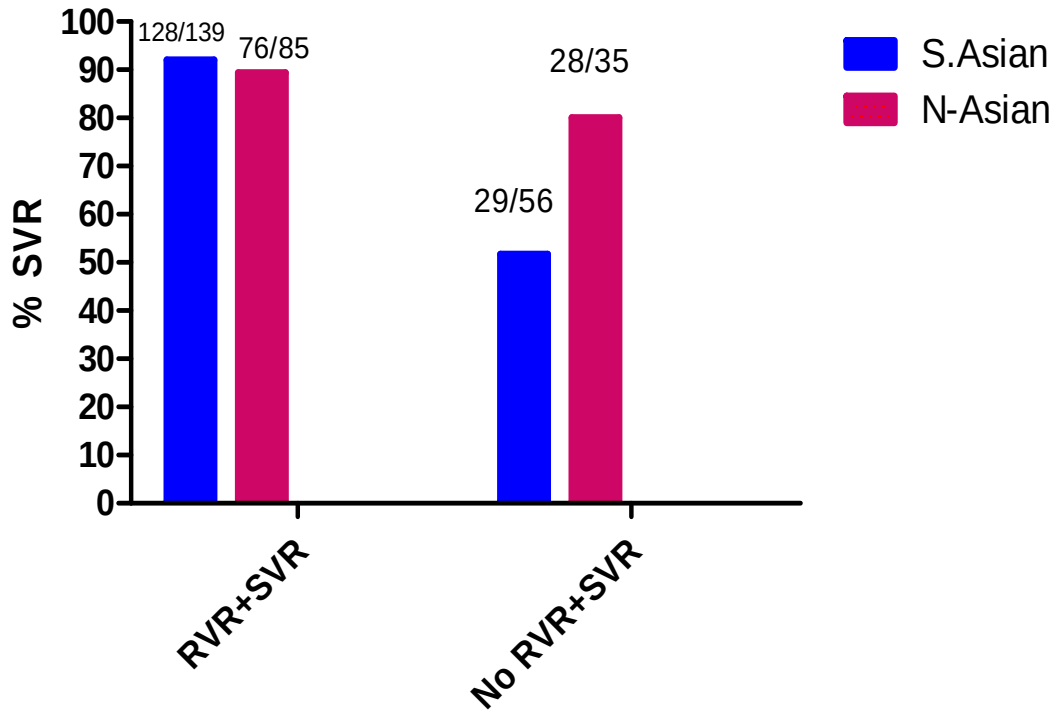
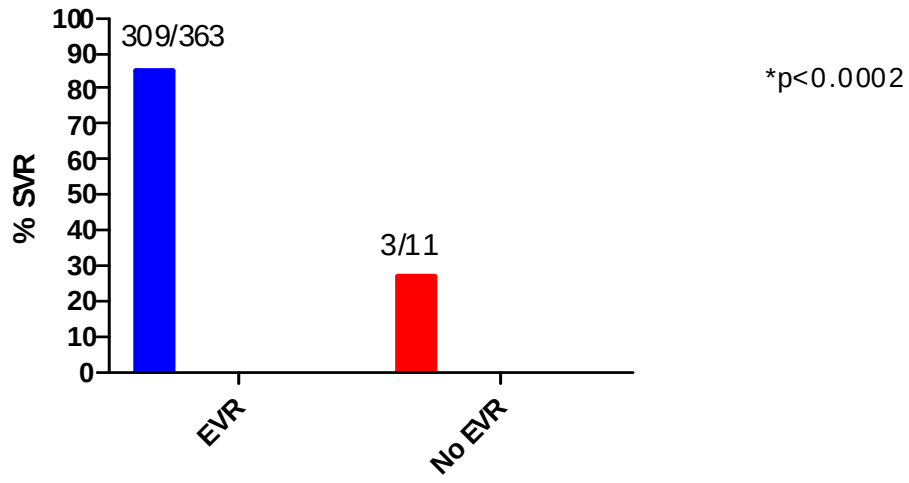


Figure 3.15 shows the results. In S. Asian patients 92.08% achieved an RVR and this was not significantly different from the proportion of non-Asian patients who achieved an RVR (89.4%) Of the S. Asian patients who achieved an RVR 92% (n=128/139) achieved an SVR and of the S. Asian patients who did not achieve an SVR 51.78% (n=29/56) attained an SVR.

In non-Asian patients a similar effect was seen - 89.41% (76/85) of patients with an RVR achieved an SVR while of the Non Asian but in Caucasian patients who did not have an RVR

62.63% (57/91) patients achieved an SVR. Hence ethnicity does not impact upon the probability of achieving an RVR and does not modify its positive value.

Figure 3.16: Impact of early virological response

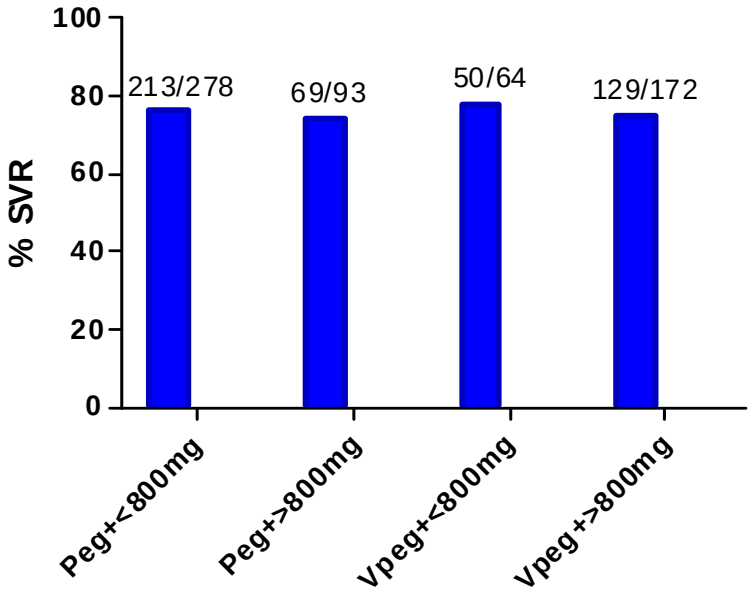


Previous studies (predominantly in patients with genotype 1 HCV) show that there is a low probability of achieving an SVR if a patient does not achieve an EVR. When looking at our genotype 3 dataset (Figure 3.16) a total of 363 patients achieved an EVR of which 85.12% (n=309/363) achieved a SVR.

In common with previous studies very few patients failed to achieve an EVR (11 of 374 tested) and the response rate was reduced in these patients to 27.2% (n=3/11). This section does not

contain all the patients as not all patients from different treatment centres had a week 12 viral load tested (EVR) and there may be a selection bias in the results from this section.

Figure 3.17: The Effect different treatment regimes on SVR

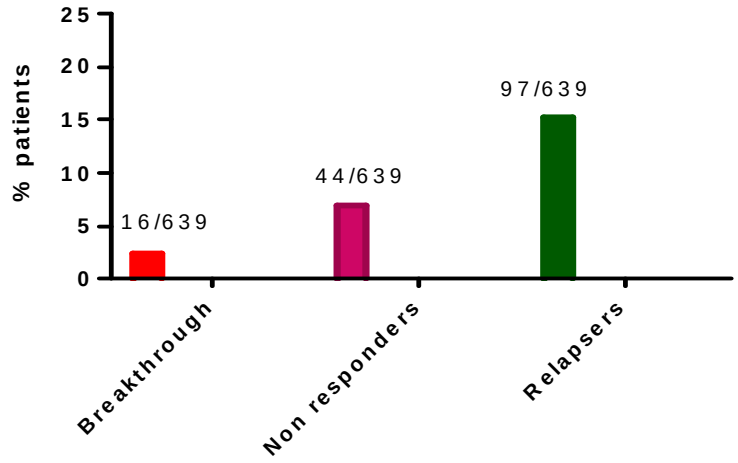


A number of different treatment regimes were used by the different groups – these included 40 kD pegylated interferon alfa 2a combined with 800 mg of ribavirin 40 kD pegylated interferon alfa 2a combined with high dose ribavirin (>1000mg); 12 kD pegylated interferon alfa 2b combined with 800 mg of ribavirin and 12 kD pegylated interferon alfa 2a combined with >1000 mg of ribavirin Figure 3.17 shows that there was no difference in response rate for any of these regimes.

Of particular note was the observation that patients treated with Pegylated Interferon alfa 2a who received a higher dose of ribavirin did not have a higher SVR rate than those receiving a lower dose. However this was not a randomized, powered comparison and confirmation of these results in an appropriately conducted study are required to confirm this observation.

The modes of treatment failure were analysed and figure 3.18 shows the results.

Figure 3.18: Modes of treatment failure in genotype 3 patients



Relapsers are defined as those patients, who experience reappearance of serum HCV RNA after achieving an undetectable level at the end of a course of therapy (an end-of treatment response), and nonresponders are those patients who do not achieve viral clearance by the completion of therapy. Some patients experience breakthrough, which is defined as an increase in HCV RNA after achieving an undetectable viral load during continuous treatment. Some have no decrease in HCV viral load during therapy, or experience only a modest decrease of 1-2 logs. Others have an HCV RNA decrease of at least 2 logs, but their viral load remains detectable during therapy.

A total of 24.56% (157 /639) patients failed to achieve an SVR. Very few patients 16 (2.5%) had a breakthrough during treatment, while 6.88% (n=44/639) were primary non responders. The major mechanism of treatment failure was relapse which occurred in 97 patients (15.17%).

Table 3.3: Full Regression analysis Model

This work was completed with the assistance of a statistician (Prof Caroline Sabin).

To analyse the multiple factors involved in the response to therapy we conducted a multivariable analysis of all of the factors. In the multivariable model age, diabetes and cirrhosis were strongly correlated and it was impossible to determine which dominated the poor response in elderly, diabetic, cirrhotic patients. In an attempt to circumvent this problem the model was run only including these factors (adjusted model) and this did not distinguish between the three variables. A further iteration was performed (adjusted model 2) in which only diabetes, cirrhosis, age and platelet count were included and, again, no distinction between them was possible. The outputs are shown in Table 3.3.

	Unadjusted OR	Unadjusted 95% CI	Unadjusted P value	Adjusted OR	Adjusted 95% CI	Adjusted P value	Adjusted model (2)[a] OR	Adjusted model (2)[a] 95% CI	Adjusted model (2)[a] P value
Male Sex	0.96	0.65, 1.42	0.83	-					
S.Asian Ethnicity	0.99	0.68, 1.44	0.97	-					
Age (>5 years older)	0.62	0.52, 0.75	0.0001	0.75	0.62, 0.92	0.008	0.81	0.65, 1.02	0.07
Year of Rx				-					
<2004	0.87	0.49, 1.56	0.64	-					
2005/2006	1.35	0.78, 2.34	0.29	-					
2007	1.14	0.63, 2.05	0.66	-					
>2008	1.0	.		-					
Cirrhosis	0.32	0.21, 0.48	0.0001	0.41	0.26, 0.65	0.0001	0.49	0.29, 0.83	0.008
Diabetes	0.39	0.23, 0.67	0.0006	0.38	0.21, 0.72	0.003	0.31	0.16, 0.61	0.0006
Rx with Pegylated IFN	1.27	0.87, 1.84	0.22	-					
Weight (> 5kg)	0.93	0.87, 1.00	0.06	N/A					
BMI (/kg/m2 higher)	0.95	0.91, 1.00	0.05	N/A					
Fibrosis score (/1 higher)	0.72	0.64, 0.82	0.0001	N/A					
ALT (/log$_{10}$	0.65	0.47, 0.91	0.01	N/A					

higher)									
Haemoglobin (/1 higher)	1.13	1.00, 1.27	0.05	N/A					
Platelets (/50 higher)	1.44	1.24, 1.66	0.0001	N/A			1.19	1.01, 1.40	0.04

As expected on treatment factors (RVR, EVR and ALT after therapy were strongly associated with a response to therapy. However ethnicity in a multivariable analysis was not associated with treatment outcome.

Discussion

An anonymised retrospective audit has been performed of patients with genotype 3 chronic HCV infection who have been treated at four UK liver units (Birmingham University Hospital, and The Royal London Hospital-Barts and the London NHS trust, Bradford Royal Infirmary ,St.Mary's/Imperial NHS Trust). A total of 639 case notes were examined and data analysed by univariable and multivariable analysis. We found an overall response rate of 74.96% in our genotype 3 patient population and we found no difference by both univariate and multivariate analysis between South Asian and Caucasian patients. Of the S.Asian patients 74.44% (n=236/317) achieved an SVR compared to 75.46% (n=243/322) of the Non Asian patients. We examined factors that may influence the outcome of therapy in patients with genotype 3 HCV and found that age, cirrhosis and diabetes had a major impact upon the response to therapy .The total number of patients included in the above dataset (639) as compared to the published paper which had 637 patients included. This was because two additional patients completed therapy after censoring the submitted paper. These additional patients were included here.

Our primary hypothesis that S. Asian patients respond less well to therapy was not confirmed by this analysis. However cirrhosis did reduce the response rate to therapy and as there is an increased prevalence of cirrhosis in the S. Asian community this may explain previous studies suggesting that ethnicity impacts upon the response. It is not yet clear whether this is because more of the older S. Asian patients had cirrhosis or whether age and cirrhosis are independent factors that influence treatment response. Future studies on the completed database will consider this important issue.

The study was retrospective in nature, it involved different drug regimes and was conducted over a long period of time. Ethnicity was self reported (as is in the NHS) so may have affected outcomes of the impact of ethnicity on response rates. The diagnosis of cirrhosis was based on various different parameters ie ultrasound scan reports,platelet counts and liver biopsy results so the diagnosis of cirrhosis was correct although heavier patients and diabetic patients in whom an ultrasound scan was indeterminate and who declined a biopsy may have been wrongly diagnosed. The use of better non-invasive markers might help us define this grey area of the population.Not many diabetics were identified in this study possibly due to diagnostic difficulties and the lack of standard insulin sensitivity testing. Dose reductions were identified from the notes but there may have been inconsistent reporting which may have reduced the value of these data. The findings with regards to response to treatment and mode of treatment failure were however; consistent with the findings of previous large scale studies with the important novel observation that ethnicity does not play a role on response to treatment. The relapse rate of the S. Asian patients was 13.35% (n=43/322) of the Non Asian patients the relapse rate was 13.35% (n=43/322). Relapse is the major mode of treatment failure seen in genotype 3 patients with an overall relapse rate of 15.17% (n=97/639) in our genotype 3 population. Future studies are needed to address the issue of how to prevent relapse in patients infected with genotype 3.

In summary, the work completed confirms the previous findings that S. Asians and Non Asians respond equally to therapy and furthermore our published study indicates that genotype 3 HCV is ‘easy to treat’ provided that the patients are identified when they are young. The factors that may affect the response rate in the patients with genotype 3 chronic HCV are diabetes, older age and cirrhosis.

CHAPTER 4

STEPS Trial

‘Study to Evaluate Pegasys SVR in Genotype 3 HCV infected Cirrhotic Patients’.

4.1 Introduction

Cirrhosis due to chronic HCV infection is one of the leading causes of liver transplantation and, sadly, patients with cirrhosis, who are most at risk of dying of liver disease, are the least responsive to therapy. Most of the studies to-date have analysed approaches to managing cirrhosis in patients with genotype 1 HCV and few studies have examined this issue in patients with other genotypes. As noted previously (see Introduction) randomised controlled trials of patients with Genotype 2 and 3 HCV have shown that 24 weeks therapy is comparable to longer durations of treatment and a number of attempts have been made to reduce the treatment duration further, with mixed success. No studies have examined prolonged exposure to interferon and ribavirin in patients with advanced fibrosis. Hadziyannis et al [155] in a retrospective subgroup analysis of a limited number of patients with cirrhosis found an SVR of 73% of genotype 2/3 patients with chronic HCV infection and did not observe any difference in response in those treated with different doses of ribavirin. This is in contrast to studies in patients with genotype 1 HCV where the dose of ribavirin had a marked effect on the response rate. In a study performed in 124 patients with advanced fibrosis and genotype 2 or 3 HCV Helbling et al compared two different doses of ribavirin combined with pegylated interferon 180ug for 48 weeks (Beat Helbling et al. 2006), they found that there was no significant difference between the two groups although patients receiving the higher dose of ribavirin (1000mg-1200mg) had a numerically higher SVR (52%) compared to 38% in those who received a lower dose. In a direct comparison of response to therapy between patients infected with genotype 3a with and without cirrhosis, those with cirrhosis had a lower SVR than those without cirrhosis 35% [6/17] versus 84%[62/74] (p<0.0005) **.** By multivariate analysis, cirrhosis was the only predictor of not attaining a SVR.

Cirrhosis might identify patients harbouring either co-factors or co-morbidities that affect IFN responsiveness. Most of the patients with cirrhosis have a longer disease duration than patients with mild liver fibrosis and are more often affected by co-morbidities such as diabetes, iron overload and alcohol abuse, which are all capable of impairing response to anti-HCV therapy.

Genotype 3 HCV is common in the Indian sub-continent and, according to recent census figures, over 2 million immigrants from this region are currently living in the UK. Anecdotal reports from a number of different units, including East London and Birmingham indicates that genotype 3 HCV is common in immigrants from Bangladesh and Pakistan. Unpublished data from East London suggests that a large proportion of these patients have advanced fibrosis. In view of the

published studies indicating that 24 weeks therapy is sufficient to eradicate genotype 3 HCV most UK units treat such patients for 24 weeks and use a standard dose of ribavirin (800mg per day). Thus in the UK a large proportion of patients with genotype 3 chronic HCV infection originate from South Asia. They often have advanced disease and they respond poorly to current therapy. It is unclear whether increasing the duration of therapy will increase the response to therapy in these patients.

To begin to address the issues around optimal therapy for patients with genotype 3 HCV we planned a multicentre, randomised clinical trial involving a comparison of 24 vs 48 weeks therapy in patients with genotype 3 HCV and advanced fibrosis. We considered evaluating different doses of ribavirin in patients with cirrhosis. However the licensed dose for Pegylated interferon alfa 2a is 800 mg per day and the study of Hadziyannis et al indicated that there was no benefit in increasing doses of ribavirin in patients with genotype 3 HCV. Given the difficulties in recruiting sufficient patients to complete an analysis of two different variables we chose to change a single variable (duration of therapy) rather than two variables and we elected to use a single, fixed dose of ribavirin in accordance with the current drug label.

4.2 Study to Evaluate Pegasys SVR in Genotype 3 HCV infected Cirrhotic Patients

This was a multicentre, open labelled, randomised study comparing 24 weeks therapy with 40 KD pegylated interferon alpha 2a and ribavirin (800 mg) (Group A) to 48 weeks therapy with 40 KD pegylated interferon alpha 2a and ribavirin (800 mg) (Group B) in patients with genotype 3 chronic HCV and advanced fibrosis (modified Ishak fibrosis score of 4 or more).

4.2.1 Trial Design

A total of 140 treatment naïve patients were randomized (1:1) at baseline to either receive 24 weeks of treatment (Group A) or 48 weeks of therapy (Group B). Both groups received 180µg Pegasys in combination with 800mg Copegus for the treatment duration. 24 weeks follow-up for both groups will be completed after treatment.

4.2.2 Primary Objectives

Our aim was to compare the efficacy and safety of 180µg dosing of pegylated interferon alfa 2a (Pegasys) in combination with ribavirin (Copegus) (800mg) for 24 weeks (Group A) with the efficacy and safety of 180µg dosing of the same drugs given for 48 weeks (Group B) in Genotype 3 HCV infected individuals with significant fibrosis or liver cirrhosis (modified Ishak

fibrosis score of equal to or greater than 4 **OR** radiological and/or endoscopic features of cirrhosis).

4.2.3 Secondary Objectives

To compare the efficacy and safety of Group A compared to Group B based on stratification of HCV RNA viral load at 4 weeks (either <50 copies/ml or ≥50 copies/ml)

To compare the efficacy and safety of Group A compared to Group B based on stratification of HCV RNA viral load at 12 weeks (either <50 copies/ml or ≥50 copies/ml)

To evaluate and compare the virological response (HCV RNA) at week 4, 24 (both groups) and week 48 (group B).

To evaluate (descriptive) baseline characteristics (baseline HCV RNA viral load, BMI, insulin resistance, fibrosis score, age and sex) and treatment outcome between Group A and B[181].

4.2.4 Centres

The trial was run as a UK multicentre study with 16 centres of which 14 centres were successful in recruiting patients. Two centres unfortunately were unable to recruit patients for the study.

4.2.5 Inclusion and Exclusion Criteria

Initially in Protocol V2 (19 Apr 2007) the minimum platelet count for inclusion in the study was >70,000 cells/mm3, Neutrophil count > 600 cells/mm3 Whereas in Protocol V3.4 (4 Feb 2010)· the entry criteria were changed to allow inclusion of patients with a lower pre-treatment platelet count (>30,000 cells/mm3) .This was done as most of the genotype 3 patients with advanced fibrosis and cirrhosis presented in our study population had a low platelet count and investigators considered this to be a safe limit for therapy and exclusion of patients with thrombocytopaenia was restricting enrollment.

A. Principal Inclusion Criteria

- Age ≥18 years of age
- Chronic genotype 3 HCV infection as evidenced by HCV antibody and RNA positivity with genotype 3 infection confirmed at a central laboratory.
- Liver biopsy within 18 months of entry showing features of chronic HCV infection and modified Ishak fibrosis score of equal to or greater than 4 OR radiological and/or endoscopic features of cirrhosis.
- No evidence of other viral infection including HBV and HIV.

- Platelet count ≥ 30,000 cells/mm^3, Neutrophil count > 600 cells/mm^3
- Compensated liver disease (Child-Pugh Grade A).

B. Principal Exclusion Criteria

- The central exclusion criteria were previous therapy for chronic HCV infection, 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, retinopathy or other severe illness (including psychiatric disorders) likely to impair compliance or to be exacerbated by interferon administration (such as autoimmune disorders) and pregnancy.

4.2.6 Randomisation procedures

Once the site had identified the patient who had fulfilled the inclusion/exclusion criteria and after the patient had signed the consent form the site telephoned the study coordinator and a randomisation number was generated by the computer which automatically assigned patients to either the 24 week or the 48 week arm.

4.2.7 Trial protocol

Details of the trial protocol are provided in the Material and Methods and the detailed Protocol is attached in Appendix 8.1.1

Results

The following are the preliminary results of an interim analysis of the trial dataset. The database lock has only just been applied so the data was "unclean"; time constraints have prevented a final analysis after the database lock but it is unlikely that the overall conclusions will differ in the final, definitive analysis, although some details may change. At the time of writing randomization and treatment of patients is complete. A few patients are still awaiting their final HCV RNA test (N=2) and others (N=8) have significant missing data that requires entry onto the e-CRF. The completed data on 131 patients was available for analysis and here I will restrict my analysis to these data. It is hoped that an update will be available to append to this thesis prior to archiving.

Figure 4.1 shows the patient disposition.

For this analysis I will restrict my analysis to the 131 total patients in whom complete data is available -64 were randomised to receive 24 weeks treatment and 67 were randomised to receive 48 weeks of treatment. Figure 3.1 shows enrolment and outcomes

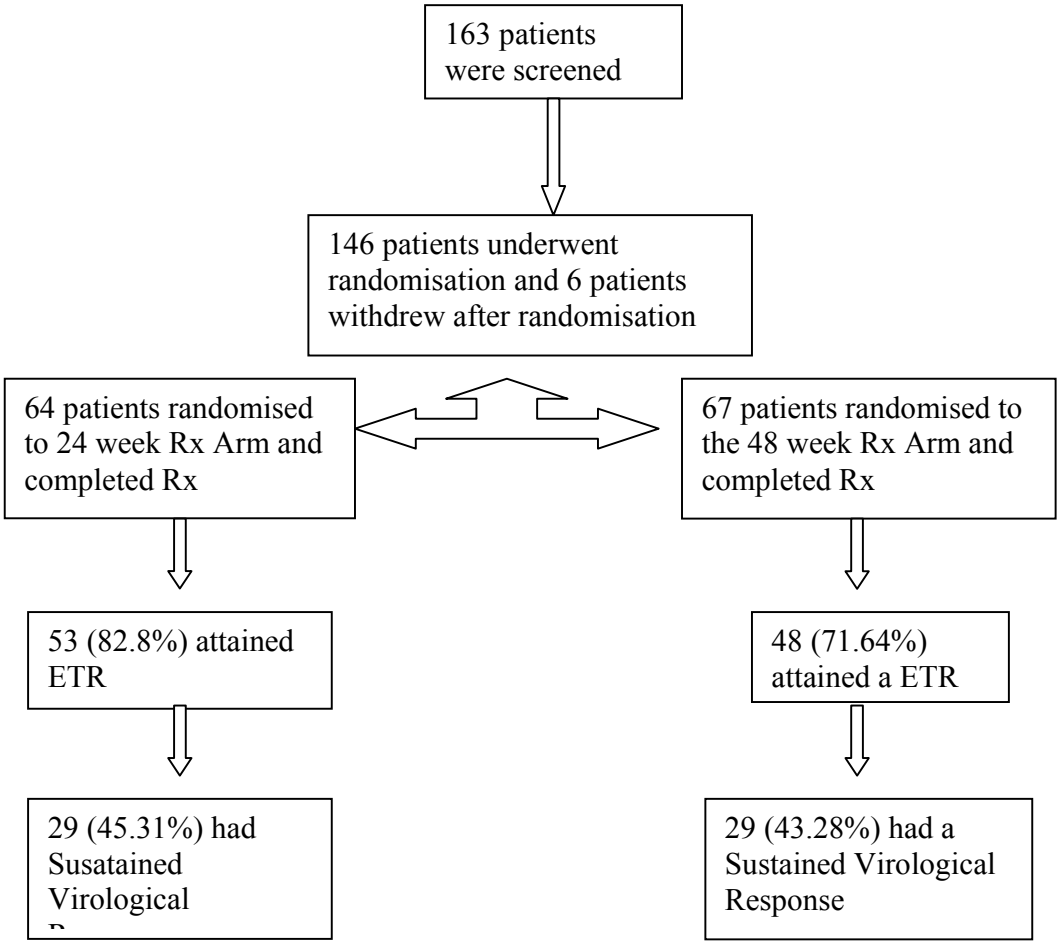


Figure 4.1 Enrolment and outcomes

Table 4.1 shows the demographic characteristics of the study population. There were no significant differences between the two arms.

Table 4.1 Baseline characteristics of patients

Factor	Randomised Trial	24 Week Arm (N=63)	48 Week Arm (N=74)
Sex	Male (64.96%)	Male (63.49	Male (65.75%)
Age mean (range)	48 (28- 74)	48.52 (33-74)	47.6 (28-66)
Ribavarin weight based dose- mg/kg mean (range)	10.65 (6.5- 17.39)	10.73 (6.5- 17.39)	10.58 (5.5- 14.81)
BMI mean (range)	27.82 (19.1- 45.3)	27.73 (19.2- 42.8)	27.9 (19.1- 45.3)
Pre Rx Hb mean (range)	14 (10.3- 18)	10.85 (10.7- 159)	20.13 (10.2- 180)
Pre Rx Platelets mean (range)	157 (37- 374)	154.64 (43- 356)	159.39 (37-374)
Pre Rx ALT mean (range)	117 (26- 423)	111.05 (27- 322)	124.12 (26-423)
Pre Rx viral load	3.39+ 0.12	2.27+0.80	4.70+0.76

Table 4.2 Withdrawals from the Study

	Randomised to 24 weeks		**Randomised to 48 weeks**		
	First 24 weeks of therapy	Withdrew from follow up	First 24 weeks of therapy	Second 24 weeks of therapy	Withdrew in follow up
Withdrawn for SAE	2	0	3	5	0
Withdrawn for lack of efficacy	8	2	5	3	1
Total withdrawals	10	2	8	8	1

There were a total of 29 patients who withdrew from the study - 17 were randomised to the 48 week treatment arm and 12 were assigned to the 24 week treatment arm. In the preliminary interim analysis there was no difference seen in the timing of patients withdrawal in the 48 week treatment arm as 8 dropped out in the initial 24 weeks and 8 dropped out in the remaining 48 weeks of treatment. More patients were withdrawn due to SAE's than lack of efficacy to treatment. Table 4.3 summarises the treatment outcome.

The overall SVR was 44.27% (58/131) and there was no significant difference between patients receiving 48 weeks of therapy (SVR = 43.28% (n-29/67) and those receiving 24 weeks of therapy (SVR = 45.31%, 29/64) $p>0.05$. The rate of relapse similarly did not differ between the two arms - 19.4% (n- 13/67) in the 48 week treatment arm and 32.81% (n-21/64) in the 24 week treatment ($p= 0.35$).

In this interim analysis the impact of RVR on treatment response was difficult to evaluate as a number of centres have not yet entered the data into the database. However in the interim analysis of the available data there was no apparent benefit of extending treatment in patients who did not achieve an RVR. In patients assigned to 24 weeks of therapy 34/64 patients (53%)

achieved an RVR and of these 24/34 patients (73%) went on to achieve an SVR. In the 29 patients who did not achieve an RVR the SVR was 17.24% .In patients assigned to 48 weeks of therapy 39/67 (58.2%) achieved an RVR and 21/39 (53.8%) went on to achieve an SVR. In the 28 patients randomised to receive 48 weeks of therapy 29% achieved an SVR and this was not significantly different from the rate seen in patients randomised to receive 24 weeks of therapy

The impact of EVR on treatment response is discussed later on in the chapter.

The End of treatment response in the 24 week treatment arm was 82.8% (N-53/64) and 71.64% (N- 48/67) in the 48 week treatment arm. This difference was not statistically significant.

The proportion of patients who did not clear HCV RNA (non-response) was small and did not differ between the two arms, being 6.25% (n-4/64) in the 24 week treatment arm as compared to 5.97% (n-4/67) in the 48 week treatment arm.

Table 4.3 : Virological Response

	24 Week Arm *no./total no. (%)*	48 Week Arm *no./total no. (%)*
Undetectable viral load at Week 4	24/34 (70.58%)	21/39 (53.84%)
Undetectable viral load at Week 12	29/64 (45.31%)	29 /67 (43.28%)
Undetectable viral load at ETR	53/64 (82.8%)	48/67 (71.64%)
SVR	29/64 (45.31%)	29/67 (43.28%)
Proportion of patients without an RVR who achieved an SVR	5/29 (17.24%)	8/28 (28.57%)
Proportion of patients with an EVR who didn't achieved an SVR	22/39 (56.4%)	17/39 (43.58%)
Relapse	21/64 (32.81%)	13/67 (19.4%)
Non Response	4/64 (6.25%)	4/67 (5.97%)

Figure 4.2 Summarises The Response To Therapy. The figure shows an SVR of 45.31% (n=29/64) in the 24 week treatment arm and a SVR of 43.28% (n= 29/67) in the 48 week treatment arm. The overall SVR in the study was 44.27% (n= 58/131). There was no significant difference between the two treatment arms (p = 0.0261)

Figure 4.2 : SVR in Different treatment Arms

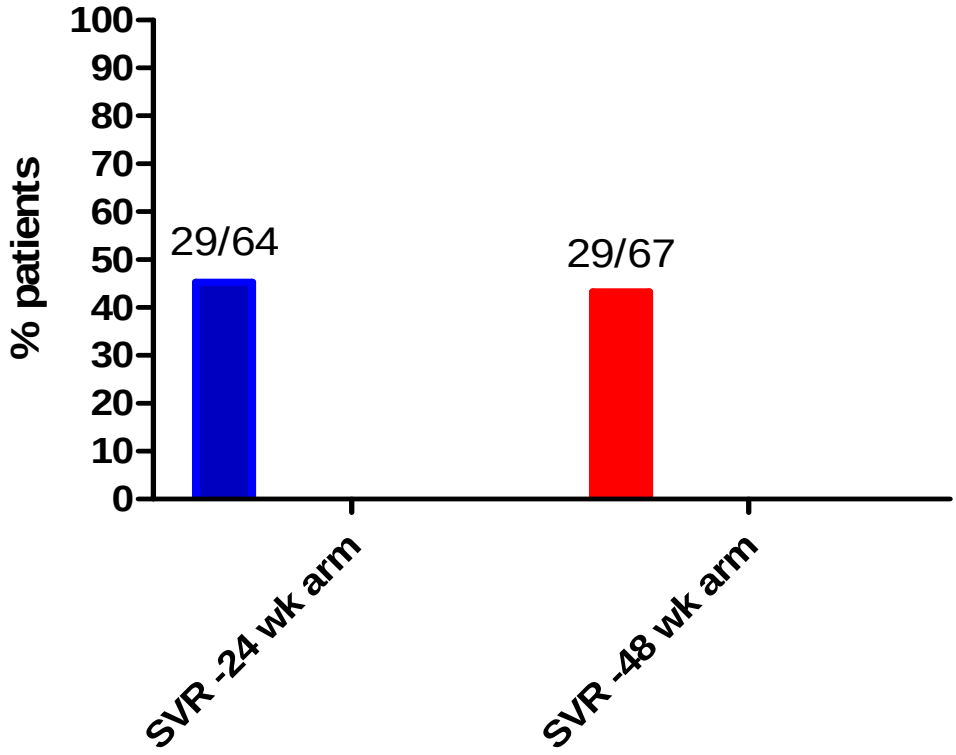
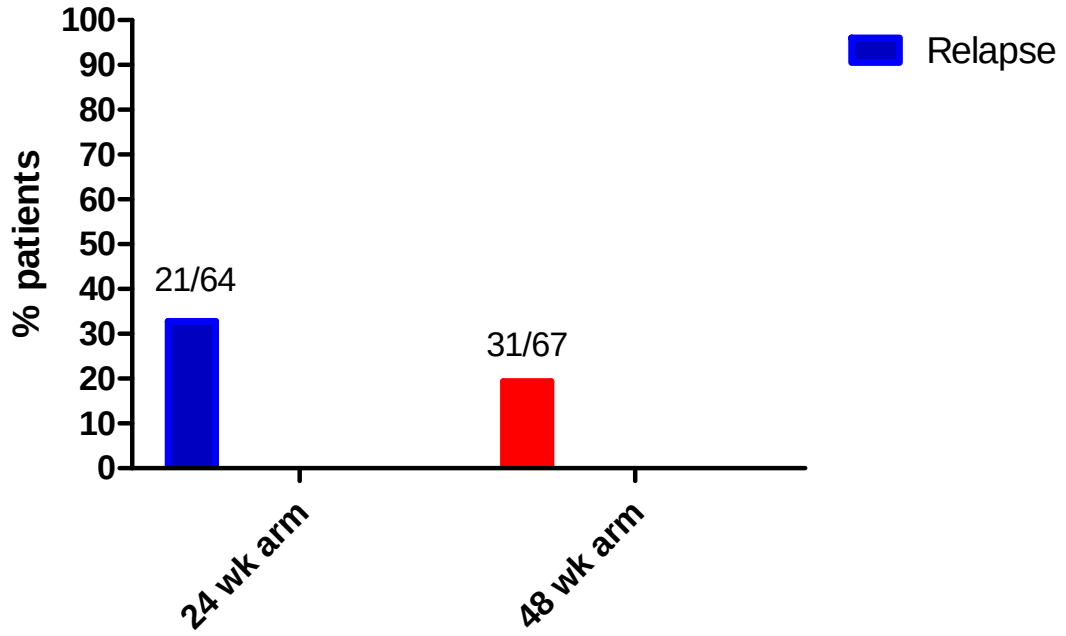


Figure 4.3 shows the difference in relapse rates between the two treatment arms. The percentage of relapse was 32.81% (n-21/64) in the 24 week treatment arm as compared to 19.4% (n- 13/67) in the 48 week treatment arm. There was a higher rate of relapse rate seen in the 24 week treatment arm but the difference was not statistically significant p = 0.35

Figure 4.3 Relapse in different Treatment Arms



Many large scale studies have shown the importance in RVR in predicting response to treatment. Of the 73 patients in the STEPS study who had an RVR 61% (n-45/73) achieved an SVR – there was no significant difference between patients with an RVR who achieved an SVR in the group receiving 48 weeks therapy (58.84%) when compared to the group receiving 24 weeks (70.6%) and although the trial was not powered to detect an increase in SVR in patients achieving an RVR the complete absence of benefit in patients with an extended duration of therapy indicates that extending the duration of therapy in patients who achieved an RVR did not prevent relapse.

There were fewer patients who did not achieve an RVR and in this group, again there was no significant advantage in SVR in patients receiving 24 or 48 weeks therapy.

Figure 4.4: Effect of RVR on Response to Treatment

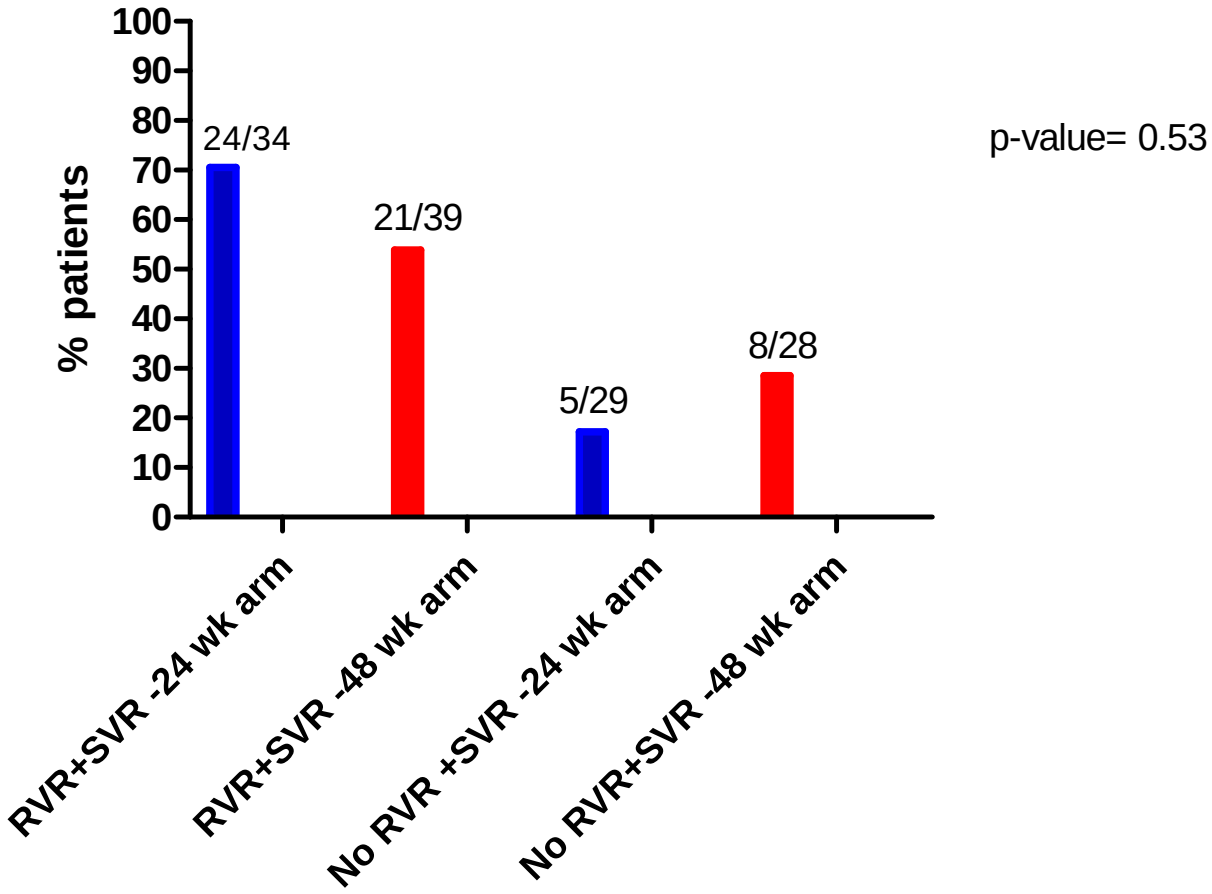


Table 4.4 Summarises the effect of EVR on response to treatment in the two treatment arms. The overall EVR in the patients who had a response to treatment was 44. 61% (n- 58/131).

In the individual treatment arms it was seen that 45.31% (n-29/64) of the patients attained an SVR in the 24 week treatment group as compared to 43.28% (n-29/67) in the 48 week treatment arm group. This difference was not statistically significant with a p value-0.861.

Table 4.4 Overall Effect of EVR on Response to Treatment

	24 week Rx Arm	**48 week Rx Arm**
EVR	64	67
EVR +SVR	29/64 (45.31%)	29 /67 (43.28%)
EVR +no SVR	22/39-56.4%	17/39 -43.58%

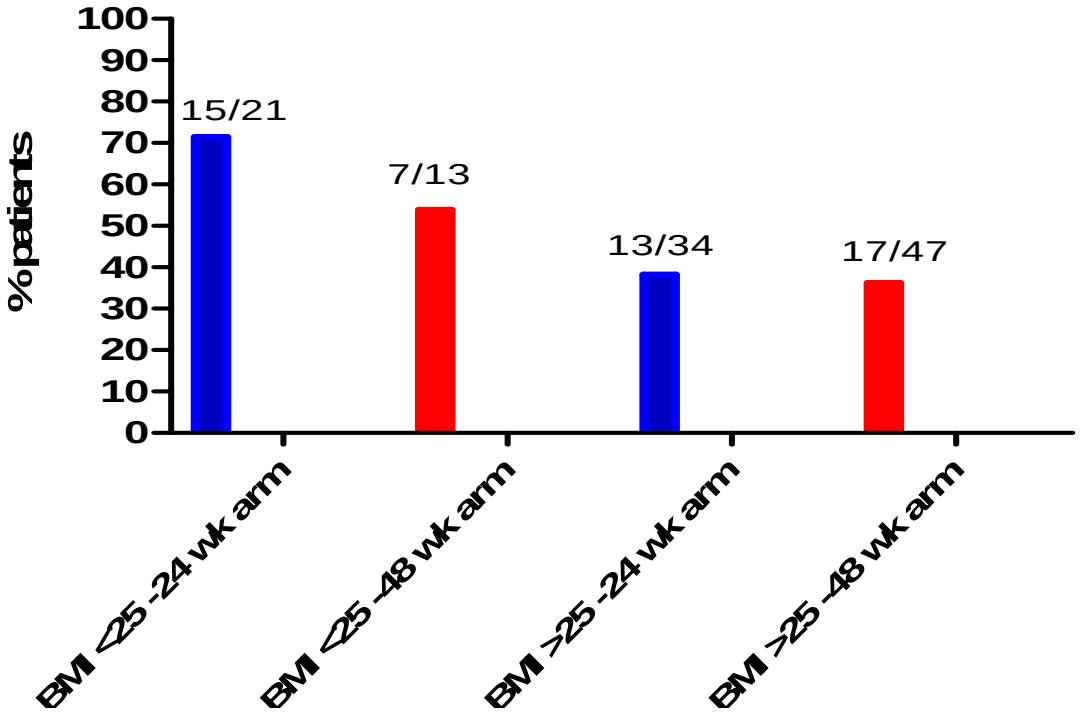
When looking at the effect of ethnicity on response to treatment in different treatment arms we found no significant difference. Of the 52 S. Asian patients 62% (n-18/29) attained an SVR on the 24 week treatment arm as compared to 65.21% (n-15/23) on the 48 week treatment arm.

Of the 45 non Asian patients 50% (n-11/22) attained an SVR on the 24 week treatment arm as compared to 65.21% (n-15/23) on the 48 week treatment arm. There was no statistically significant difference seen and response to therapy was not influenced by ethnicity.

Figure 4.5 looks at the effect of BMI on response to treatment on different treatment arms. A BMI >25 was classified as overweight and a BMI <25 was classified as normal.

A total of 82 patients had a BMI of >25 and 35 patients had a BMI <25. Of the overall 82 patients who had a BMI>25 36.58% (n-30/82) attained an SVR as compared to 62.85% (n-22/35) in the patients who had a BMI<25.This difference was statistically significant (p =0.0142) signifying that heavier patients are less likely to achieve an SVR. However the number of patients studied was relatively small and the trial was not powered to detect a difference in BMI and treatment outcome.

Figure 4.5: BMI and its effect on SVR in different Treatment Arms



Given the uncertainty surrounding the optimal dose of ribavirin in patients with advanced fibrosis and genotype 3 HCV and given that our BMI observations (above) have shown that patients who are heavier and have a higher BMI don't respond as well to treatment as patients who have a lower BMI we conducted a preliminary, hypothesis generating examination in which we compared light weight patients (who received a dose of ribavirin of > or equal to10.66mg/kg) with heavier patients and we evaluated the differences in response to treatment.

Patients who received a higher weight based dose of ribavirin did slightly better on treatment than patients who were heavier and thereby received a lower weight based dose of ribavirin. This difference was, however, not found to be statistically significant, although further studies with larger cohorts of patients will be required to determine whether or not there is any benefit of increasing the dose of ribavirin in patients who are heavier.

Discussion

This is the first randomised study of genotype 3 cirrhosis with extended therapy. No difference was seen and although not all the data is complete it seems unlikely that the final outcome will differ markedly from the data presented here.

The drop out rate was found to be higher than expected in this trial of patients with advanced liver disease. However other studies in patients with advanced cirrhosis have noted similar high levels of treatment discontinuation and since a large proportion of patients discontinue therapy it is clear that attempts to improve response rates by extending the course of treatment will not succeed.

Given that a small number of patients who did not achieve an RVR were studied we can not exclude the possibility that nonRVR patients may benefit from extended duration of therapy but given the data here we think this is unlikely and the high rate of treatment withdrawals during the second 6 months of therapy suggest that there are unlikely to be major benefits in this group of patients.

It is interesting to speculate on how the virus can be undetectable for 44 weeks in patients complying with therapy and yet still relapse. As discussed in Chapter 6 the development of novel viral quasispecies might be linked to an unusual pre-treatment variant perhaps an interferon resistant viral strain that replicates at low levels during treatment and then re-emerges once therapy is discontinued. Alternatively there may be a 'sanctuary site' where low level replication can continue during therapy and then emerge when treatment is discontinued.It would be interesting to see the response to treatment in Genotype 3 non RVR patients.

In summary this trial indicates that extending the duration of therapy with interferon and ribavirin is unlikely to be of major benefit in patients with genotype 3 HCV and cirrhosis and it will be interesting to see if similar patterns emerge from studies with the new direct acting antiviral agents.

Chapter 5

Performance of Surrogate Markers of liver fibrosis in S.Asian patients with genotype 3 HCV

5.1 Introduction

Liver biopsy is considered as the gold standard for assessing hepatic fibrosis. It serves as an important tool for physicians treating patients with chronic liver disease as it provides both diagnostic and prognostic information. Prognostic information is derived from the amount of fibrosis in the liver, it is generally accepted that patients with more fibrosis are more likely to develop complications in the near future. However liver biopsy is frequently declined by patients, as being an invasive procedure associated with complications, which are rare. It is expensive and prone to sampling error and the histological analysis of liver biopsy may be prone to intraobserver and interobserver variation. This has led to the development of surrogate non invasive biomarkers of fibrosis which are biochemical parameters identified in the patients serum and are believed to correlate with the stage of fibrosis progression within the liver[179, 191].The markers are further classified into two categories as mentioned previously: **Direct Markers-** these are parameters reflecting the actual fibro genesis and the extra cellular matrix turnover and **Indirect Markers –** these are based on altered liver function tests observed in clinical practice. Within each of these groups a several different markers are combined to create a unique test with enhanced efficacy [191].

The Enhanced liver fibrosis test (ELF) was developed by Rosenberg et al [1] who studied liver biopsies obtained from 1021 predominantly Caucasian patients with a mixed pool of chronic liver disease- 496 patients had chronic HCV infection. The ELF test comprises of a panel of three direct serum markers of extra cellular matrix turnover (ECM) these include hyaluronic acid, pro collagen III amino terminal peptide (PIIINP) and tissue inhibitor of the matrix metalloproteinase (TIMP-1).This study looked at the validity of the ELF test and compared it with the liver biopsies .The biopsy specimens and serum samples were available for 921 subjects in the final analysis group. The test cohort and the validation cohort were derived from this group.To derive the algorithms combining serum markers, a group of 400 cases from the test cohort were selected at random from the group of 921 patients with biopsy specimens. An optimal algorithm was selected and the performance of this algorithm was then validated in the remaining set of 521 biopsy specimens from the final group, designated as the validation cohort[1].Based on these specific markers the European Liver Fibrosis group developed an

algorithm that can measure these different assays to give a score known as the ELF score which can be correlated to the different stages of fibrosis.The algorithm classifies a score of 6.6 -7.4 as mild disease, a score of 7.4-10.5 as moderate disease, a score of 10.5-12 as moderate severe and a score of >12 as cirrhosis.

Disease	AUC	Score	Sensitivity	Specificity	PPV(%)	NPV(%)
NAFLD	0.870	0.375 0.462	89% 78%	96% 98%	80% 87%	98% 96%
ALD	0.944	0.087 0.431	100% 93.3%	16.7% 100%	75% 100%	100% 85.7%
HCV	0.773	0.067 0.564	90% 30%	31% 99%	27.5% 89.5%	92.3% 83.3%

Table 5.1 - Performance of ELF test [1]

Since its development the test has been used in a number of studies and shown to be valuable in PBC and in children with NASH and apart from the original cohort and the 112 paediatric patients with NAFLD were studied [1, 192].

Various other tests have been used to define the severity of Liver fibrosis. The FIB-4 test combines standard biochemical values (platelets, ALT, AST) and age and is used as an inexpensive method to assess liver fibrosis. The FIB-4 test was first introduced by the APRICOT group who showed that the test had an area under the receiver operating characteristic (ROC) curve of 0.76 , a value of <1.45 had a negative predictive value for extended fibrosis (F4-F6 in the Ishak classification) of 90% and a value of >3.25 was found to have a positive predictive value for extended fibrosis/cirrhosis of 65% [193].In a study done by Vallet Pichard et al in which 847 liver biopsies were performed in HCV patients a FIB-4 index outside 1.45-3.25 was associated with severe fibrosis and cirrhosis (F3-F4) with an area under the receiver operating characteristic curve of 0.85 (95% CI 0.82-0.89) and 0.91 (95% CI 0.86-0.93) [194]. Forns et al studied a group of 476 patients and found that age, gamma glutamyl transpeptidase (GGT), cholesterol, platelet count, and prothrombin time were independent predictors of fibrosis. The group devised a scoring system which combines age, GGT, cholesterol, and platelet count that was useful to identify patients without significant hepatic fibrosis. A cut off score <4.2 excluded patients with significant fibrosis (F2 to F4) could be excluded with high accuracy (negative predictive value of 96%) in 125 (36%) of 351 patients [195]. Platelet count and AST level were found to be important predictors of both significant fibrosis and cirrhosis. Wai et al studied 270

patients with chronic HCV infection and divided them into 2 sequential cohorts: training set (n=192) and validation set (n= 78).
A novel index, AST to platelet ratio index (APRI), was developed to amplify the opposing effects of liver fibrosis on AST and platelet count. The AUC of APRI for predicting significant fibrosis and cirrhosis were 0.80 and 0.89, respectively, in the training set. Using optimized cut-off values, significant fibrosis could be predicted accurately in 51% of the patients and cirrhosis in 81% of patients. The AUC of APRI for predicting significant fibrosis and cirrhosis in the validation set were 0.88 and 0.94, respectively[196].

The Fibrotest combines serum concentrations of ά-2 macroglobulin, haptoglobin, γ-GT, bilirubin, and apolipoprotein A1. Fibrotest is by far the most investigated and validated non invasive marker of liver fibrosis with around 20 studies reported in the literature. It has been extensively tested in chronic hepatitis C where it shows an AUC of around 0.85 for significant fibrosis[197].Fibrotest has also been tested in chronic hepatitis B and it performed slightly worse, with an AUC of 0.78 in the only study conducted[179]. In the only study performed in HIV/HCV co-infected patients fibrotest performed well, particularly for cirrhosis (AUC = 0.87) that could be excluded with 100% negative predictive value. Studies have shown that fibrotest performs well in detecting the two extremes of the staging range of liver fibrosis (F0-1 and F4), while it might performs somehow less well in the intermediate stage (F2). Fibrotest was also found to predict fibrosis in alcoholic and non-alcoholic fatty liver disease [179, 198]. The diagnostic accuracy of this test is limited by hemolysis (leading to a reduction in haptoglobin), Gilbert's syndrome (increasing the bilirubin level), and recent or ongoing infection (leading to elevations of α2-macroglobulin and haptoglobin)[198, 199].

Radiological techniques such as ultrasound, CT scan or MRI can accurately detect cirrhosis at advanced stages. Transient elastography by Fibroscan is a promising new technique that estimates the degree of hepatic fibrosis by measuring liver stiffness.In cirrhotic patients, liver stiffness measurements (LSM) show a wide range from approximately 12 to 75 kPa[179]. A study in patients with chronic HCV infection and persistently normal transaminases reported values of 100% for sensitivity, specificity, PPV and NPV, respectively. In a study of 711 patients with chronic liver disease of various etiologies, LSM was closely related to fibrosis stage

(r = 0.73) and high diagnostic accuracy was found for the diagnosis of cirrhosis (AUROC 0.96).Ganne-Carrie et al further elaborated LSM for diagnosing cirrhosis and confirmed high diagnostic accuracy (AUROC 0.95) at a cut-off value of 14.6 kPa for cirrhosis**.** Kettaneh A et al evaluated the success rate and performance of fibroscan in 935 patients with chronic HCV infection and reported successful measurements in 97% of the patients [200]. Vizzutti F et al looked at the correlation between liver stiffness measurement and portal pressure as estimated by the hepatic venous pressure gradient (HVPG) in patients with HCV related cirrhosis. The group reported a good correlation between liver stiffness measurement and high venous pressure gradients (r = 0.82) at lower portal pressures (<12 mmHg) and at a cut-off of 13.6 kPa, LSM could reliably predict or exclude clinically significant portal hypertension (CSPH, i.e. a portal pressure of ≥ 10 mmHg)[201] .Thus, fibroscan may be the method of choice to characterise the severity of cirrhosis and to identify patients with high portal pressures. This approach should be looked at in future studies[202, 203]. However, these techniques fail to detect earlier stages of hepatic fibrosis or cirrhosis. Factors that may confound or prevent the use of fibroscan are hepatic steatosis, obesity, ascites or in patients with narrow intercostal spaces[179, 203].

All serological markers have been developed in Caucasians and there is no available data relating to the validity in other racial groups. Our studies on treatment response in patients from South Asia have shown the importance of the diagnosis of cirrhosis in determining treatment outcome and we therefore examined the value of published non invasive tests in this population.

5.2 Evaluation of non-invasive markers in non Caucasian patients with genotype 3 HCV

A cohort of patients was selected (see Materials and Methods) and used to evaluate a number of non-invasive tests of liver fibrosis. The following table shows the patient demographics. 68 S. Asian patients were identified retrospectively – all had a liver biopsy within the past 3 months and had serum samples stored in the Department of Virology. These patient's biochemical/haematology parameters (i.e. AST, ALT, Platelet count, GGT) were also recorded on samples taken at the time of biopsy.

Table 5.2: Patient Demographics

Patient ID	Pts Age	AST	ALT	GGT	Platelet	Ethnicity
RLH001	15	65	63	35	144	S. Asian
RLH002	27	35	19	37	212	S. Asian
RLH003	29	106	28	30	135	S. Asian
RLH004	29	178	66	308	29	S. Asian
RLH 005	30	79	33	34	266	S. Asian
RLH 006	31	98	31	34	196	S. Asian
RLH 007	32	34	15	45	216	S. Asian
RLH 008	32	63	12	45	366	S. Asian
RLH 009	33	41	14	18	354	S.Asian
RLH 010	33	56	31	11	302	S.Asian
RLH 011	33	61	19	30	241	S.Asian
RLH 012	33	133	31	79	162	S.Asian
RLH 013	33	56	17	30	183	S.Asian
RLH 014	34	40	8	31	376	S.Asian
RLH 015	34	56	16	114	167	S.Asian
RLH 016	35	35	12	11	207	S.Asian
RLH 017	36	28	9	.	305	S.Asian
RLH 018	36	58	16	.	251	S.Asian
RLH 019	36	101	23	25	168	S.Asian
RLH 020	36	43	30	30	195	S.Asian
RLH 021	37	51	19	72	208	S.Asian
RLH 022	38	73	16	22	277	S.Asian
RLH 023	39	74	15	28	195	S.Asian
RLH 024	40	43	18	73	241	S.Asian
RLH 025	40	30	19	72	277	S.Asian
RLH 026	41	52	39	61	220	S.Asian
RLH 027	41	120	24	81	141	S.Asian
RLH 028	41	194	36	336	145	S.Asian
RLH 029	41	74	21	73	169	S.Asian
RLH 030	41	97	20	36	202	S.Asian
RLH 031	43	36	13	49	184	S.Asian
RLH 032	44	30	8	123	251	S.Asian
RLH 033	44	84	9	202	187	S.Asian
RLH 034	44	19	13	22	269	S.Asian
RLH 035	44	117	19	40	99	S.Asian
RLH 036	45	80	13	40	104	S.Asian

Table 5.2: Patient Demographics (Continue...)

RLH 037	45	47	14	.	241	S.Asian
RLH 038	45	60	6	33	282	S.Asian
RLH 039	46	36	6	56	226	S.Asian
RLH 040	47	20	23	95	286	S.Asian
RLH 041	48	95	39	98	225	S.Asian
RLH 042	49	151	16	118	186	S.Asian
RLH 043	49	52	6	15	165	S.Asian
RLH 044	50	101	15	73	168	S.Asian
RLH 045	50	16	10	31	358	S.Asian
RLH 046	50	141	108	42	341	S.Asian
RLH 047	51	86	6	73	280	S.Asian
RLH 048	51	41	16	43	281	S.Asian
RLH 049	54	23	18	91	122	S.Asian
RLH 050	55	63	16	101	232	S.Asian
RLH 051	55	45	6	38	55	S.Asian
RLH 052	55	105	13	31	292	S.Asian
RLH 053	57	44	31	33	288	S.Asian
RLH 054	57	117	21	130	83	S.Asian
RLH 055	58	84	10	252	187	S.Asian
RLH 056	60	51	18	.	219	S.Asian
RLH 057	62	66	23	457	136	S.Asian
RLH 058	63	106	28	92	135	S.Asian
RLH 059	63	72	16	14	258	S.Asian
RLH 060	66	117	25	206	81	S.Asian
RLH 061	67	88	17	57	81	S.Asian
RLH 062	68	157	16	227	105	S.Asian
RLH 063	70	77	13	22	139	S.Asian
RLH 064	70	66	14	134	134	S.Asian
RLH 065	76	30	17	30	231	S.Asian
RLH 066	25	21	28	13	225	S.Asian
RLH 067	41	26	19	53	245	S.Asian
RLH 068	53	58	8	25	64	S.Asian

Table 5.2- shows the patient demographics and the data collected of 68 S.Asian patients of Pakistani origin. Our goal was to look at the validity of the different surrogate markers and compare them to the gold standard liver biopsy in the S. Asian genotype 3 patient populations.

Table 5.3 : Different Scores

Patient ID	Fibrosis Score	APRI score	Elf Score	AST/ALT	FIB-4
RLH001	6	1	9.82	1	0.107
RLH002	6	0.36	7.48	1.84	0.23
RLH003	6	1.74	10.63	3.78	0.81
RLH004	6	13.6	13.35	2.69	2.69
RLH 005	6	0.65	9.26	2.39	0.26
RLH 006	6	1.1	8.78	3.16	0.5
RLH 007	6	0.34	8.81	2.26	0.33
RLH 008	6	0.38	9.66	5.25	0.45
RLH 009	6	0.25	5.59	2.92	0.27
RLH 010	6	0.41	9.06	1.80	0.19
RLH 011	6	0.56	8.44	3.21	0.43
RLH 012	6	1.8	10.26	4.29	0.87
RLH 013	6	0.6	9.74	3.29	0.59
RLH 014	6	0.23	6.69	5	0.45
RLH 015	6	0.74	10.11	3.5	0.71
RLH 016	6	0.37	8.20	2.91	0.49
RLH 017	6	0.2	9.56	3.11	0.36
RLH 018	6	0.51	8.97	3.62	0.33
RLH 019	6	1.2	10.84	4.39	0.94
RLH 020	5	0.49	8.19	1.43	0.26
RLH 021	5	0.54	9.00	2.68	0.47
RLH 022	5	0.58	6.37	4.56	0.62
RLH 023	5	0.84	7.90	4.93	0.98
RLH 024	5	0.39	9.66	2.38	0.39
RLH 025	5	0.24	7.59	1.57	0.22
RLH 026	5	0.52	9.93	1.33	0.027
RLH 027	5	1.89	10.58	5	1.45
RLH 028	5	2.9	12.29	5.38	1.52
RLH 029	4	0.97	9.23	3.52	0.85
RLH 030	4	1.06	6.89	4.85	0.98
RLH 031	4	0.1	7.75	2.76	0.64
RLH 032	4	0.26	9.94	3.75	0.65
RLH 033	3	0.63	10.31	9.33	2.19
RLH 034	3	0.15	8.21	1.46	0.23
RLH 035	3	2.6	8.60	6.15	2.73
RLH 036	3	1.7	10.93	6.15	2.66
RLH 037	3	0.43	10.40	3.35	0.31

Table 5.3 : Different Scores (Continue..)

RLH 038	3	0.47	10.88	10	1.59
RLH 039	3	0.29	8.46	6	1.22
RLH 040	2	0.15	9.67	0.86	0.14
RLH 041	2	0.93	10.95	2.43	0.51
RLH 042	2	1.8	9.62	9.43	2.48
RLH 043	2	0.7	8.65	8.66	2.57
RLH 044	2	1.3	11.82	6.73	2.00
RLH 045	2	0.09	7.73	1.6	0.22
RLH 046	2	0.9	10.78	1.30	0.19
RLH 047	2	0.68	12.08	14.33	2.61
RLH 048	1	0.32	8.47	2.56	0.46
RLH 049	1	11.7	11.31	1.27	0.56
RLH 050	1	0.6	10.53	3.93	0.93
RLH 051	1	1.8	9.26	7.5	7.5
RLH 052	1	0.79	9.15	8.07	1.52
RLH 053	1	0.33	9.05	1.41	0.28
RLH 054	1	3.1	12.24	5.57	3.82
RLH 055	1	0.99	11.51	8.4	2.60
RLH 056	1	0.51	10.78	2.83	0.73
RLH 057	1	1.07	12.56	2.86	1.30
RLH 058	1	0.25	6.78	3.78	1.76
RLH 059	1	0.62	8.46	4.5	1.09
RLH 060	1	3.2	12.97	4.68	3.81
RLH 061	1	5.1	11.38	5.17	4.28
RLH 062	1	3.3	12.48	9.8	6.35
RLH 063	1	1.23	7.92	5.92	2.98
RLH 064	1	1.09	11.06	4.71	2.46
RLH 065	1	0.28	9.32	1.76	0.58
RLH 066	1	0.2	8.31	0.75	0.083
RLH 067	0	0.23	8.34	1.36	0.22
RLH 068	0	2.01	9.19	7.25	6.00

Table 5.3 shows the liver biopsy scores with the different surrogate markers i.e. the APRI, ALT/AST ratio, ELF score and FIB-4.

Table 5.4: Performance of ELF test (different parameters)

Threshold Value (F5/6)	Sensitivity	Specificity	PPV	NPV
12.0	0.43	0.59	0.11	0.90
10.5	0.41	0.98	0.92	0.74
9.65	0.82	0.83	0.76	0.87
9.15	0.89	0.66	0.63	0.90
8.0	1.0	0.27	0.47	0.10

Table 5.4 demonstrates the performance of the ELF test in our study population. By using different cut off thresholds in accordance with the published normative range of ELF in which a score of >12 signifies cirrhosis we found that the ELF test was able to identify 5/19 of our patients with cirrhosis. When the normative range for identifying cirrhosis was taken as 9.65 it correctly identified 82% of patients with cirrhosis (modification of the original ELF criteria kindly performed by Dr J. Parkes). These data indicate that in this population the ELF test as originally defined performs poorly but a modification to the cut-off value significantly improves the performance.

Table 5.5: Correlation between different Surrogate markers

Fibrosis (F-6 versus F0-5) **Fibrosis (F-5/6 versus F 0-4)**

	Sensitivity	Specificity	PPV	NPV	Sensitivity	Specificity	PPV	NPV	R2
ELF (>9.65)	0.46	0.88	0.78	0.65	0.68	0.83	0.78	0.75	0.057
APRI	0.11	0.69	0.05	0.83	0.22	0.55	0.07	0.82	0.0059
AST/ALT	0.30	0.80	0.84	0.24	0.41	0.60	0.78	0.22	0.4016
FIB-4 (>3.25)	0.00	0.698	0.00	0.87	1.00	0.64	0.21	1.00	0.2251

We tested different surrogate markers in our genotype 3 patient cohort and compared them with liver biopsy. The surrogate markers as shown in table 4.5 were ELF, APRI, ALT/AST and FIB-4 were tested against Fibrosis score F6 versus F0-5 and Fibrosis score F5/6 versus F 0-4. As shown above all of the non-invasive tests performed less well in the S. Asian patients with genotype 3 HCV. These data indicate that new, more effective non-invasive markers of liver fibrosis may be equired to accurately diagnose cirrhosis in patients with genotype 3 HCV who originate from S. Asia

Discussion

In this retrospective cohort study, we compared Ishak liver fibrosis score with different surrogate marker tests i.e. ELF,FIB-4, APRI, ALT/AST ratio in 68 patients of South Asian ethnicity with genotype 3 HCV infection. Serological tests were performed on stored samples taken within three months of biopsy.

The median fibrosis score was 3 (0-6) and 41% of patients had cirrhosis on liver biopsy. ELF scores correlated well with fibrosis score (Spearman rank correlation coefficient 0.67, $p < 1^{-10}$ and the area under receiver-operator curve (AUROC) without assumptions about discriminant thresholds for fibrosis stage was 0.88 (0.81, 0.96). However the standard ELF threshold of 12.0 for cirrhosis yielded lower sensitivity, specificity, PPV, NPV compared to published series of largely Caucasian patients. Analysis of a range of ELF threshold values for cirrhosis suggests that 9.65 was the optimum value to discriminate between South Asian patients with genotype 3 HCV infection who have advanced fibrosis / cirrhosis (F5-6/6) and those who do not.

Other surrogate marker tests were also assessed in our patient population. Samples were sent to be tested for the Fibrotest but unfortunately as our serum samples were stored at -20 degrees and were exposed to light many of the constituents i.e. bilirubin ,AST were degraded so proper analysis of the samples wasn't possible and our test results were inconclusive.

Of the four surrogate markers that have been tested in our patient population i.e. APRI, AST/ALT, FIB-4 the ELF score had a better correlation coefficient of R2=0.0575 as compared to AST/ALT (R2=0.4016), APRI (R2=0.0059) and FIB-4 (R2=0.2251) in identifying patients with advanced fibrosis/cirrhosis. The other surrogate marker tests were published and validated in Caucasian cohorts but did not perform well in our cohort of patients and may need their cut-off's for detection of advanced fibrosis/cirrhosis revised in patients of S. Asian ethnicity.

Our data has shown that in 68 patients with chronic genotype 3 HCV infection correlation between ELF and histology scores remains significant, but that the thresholds for the detection of advanced should be revised in individuals of South Asian ethnicity. These data require validation in an independent cohort of patients.

The aim of this work was to identify the best marker of fibrosis in patients from S. Asia with genotype 3 HCV. We were surprised to discover that all of the tested markers performed poorly in this population. It is unclear whether the difficulties noted are due to the genotype of the infecting virus (it is conceivable that algorithms for non-invasive markers work poorly in genptype 3) or whether the assay failures are a feature of S. Asian patients of all genotypes. It is clear from our data the much more work is needed in this area to evaluate and refine current markers of fibrosis. Specifically there is a need to conduct large scale studies in patients from S. Asia with different genotypes and a need to perform large studies in cohorts of patients with genotype 3 HCV. Such studies should be analysed by ROC approaches (not pursued in our studies because of the relatively small number of patients included in the studies).

Chapter 6

Viral and Immunological Analysis of Virological Relapse in Patients with Chronic HCV Genotype 3

6.1 Introduction

This chapter describes preliminary experiments to analyse some of the virological and immunological features associated with relapse in patients with genotype 3 HCV.

6.1.1 Role of Treg cells in HCV

Natural $CD4^+CD25^+$ Treg cells were first defined in 1995 by Sakaguchi and colleagues, who showed that the transfer into athymic nude mice of lymphoid cell populations from which CD4+ T cells expressing the α-chain of the IL-2 receptor (IL-2R alpha; also known as CD25) had been removed caused spontaneous development of various T-cell-mediated autoimmune diseases. Furthermore, reconstitution with CD4+CD25+ T cells prevented the development of autoimmunity[204].

$CD4^+CD25^{high}$ Tregs constitute an important component of the immune system. About 2%-5% of the peripheral $CD4^+$ T cells in normal individuals are $CD4^+$ $CD25^{high}$ Treg cells [37]. T regulatory cells are potent suppressors of $CD4^+$ and $CD8^+$ T cells in vivo and in vitro. The natural T regulatory cells which are derived from the thymus ($CD4^+$ $CD25^+$ $Foxp3^+$) are important for the maintenance of self tolerance and to control autoimmunity[37]. Walker et al showed that the $CD25^+$ $CD4^+$ T cell subset in peripheral blood lymphocytes which express Foxp3 are capable of suppressing the activation and expansion of other T-cells in vitro as shown in rodent models[10]. Activation of CD25- CD4+ T cells by T cell receptor stimulation induces Foxp3 expression and those Foxp3 expressing CD25- CD4+ T cells are equally suppressive as natural T regulatory cells [204]. Since CD25 is expressed by both activator and regulatory T cells however, $CD25^+$ Tregs may be more specifically defined by the presence of the intracellular signalling molecule, Foxp3 and a number of studies have examined Foxp3 expressing T cell in chronic HCV and have shown that $CD4^+CD25^{high}$ and $Foxp3^+$ Tregs are upregulated in HCV infected subjects[205].

Foxp3 is a specific marker for naturally occurring regulatory T cells (nTreg). Foxp3 expression is important in the differentiation and function of nTregs. Studies in mice carrying a mutation in the Foxp3 gene have been crucial to understand the relationship of expression of Foxp3, the occurrence of nTreg and autoimmunity. The *scurfy* mutation in mice is associated with a null mutation of the Foxp3 gene, resulting in complete loss of nTregs, autoimmunity and premature death[8]. Roncarolo et al. (2008) demonstrated that Foxp3 mutations in humans lead to a lack of nTreg which is consistent with data from mice[8]. In this context it is important to note that in murine models of HSV infection elimination of Foxp3 positive cells led to an increase in viraemia, perhaps suggesting that Foxp3 positive cells have roles other than suppression of immune responses[206].

Many studies have shown that Tregs are abundant in HCV. In patients with chronic HCV infection there are elevated circulating frequencies of Tregs that may impair the HCV specific response, leading to suggestions that activation of Tregs is one of the mechanisms used by HCV to down regulate the antiviral immune response [31]. However Godkin et al, (2008) showed that Treg cells with specificity for HCV appear to be induced after viral clearance[26]. The fact that a population of Treg cells which are capable of regulating anti-HCV responses can be found after successful eradication of the virus suggests that they may not be essential for the maintenance of viraemia. It is unclear whether the development of Tregs following viral clearance is due to a reaction to the virus eliminating immune response that limits 'bystander' damage or to a direct role of Tregs in viral clearance [26].

The role of Foxp3 Tregs in patients with chronic HCV is thus controversial with suggestions that they are responsible for the persistence of viraemia and suggestions that they may be associated with viral clearance. Unpublished work from our group has studied Foxp3 positive cells during therapy. We find that, in patients with genotype 3 HCV undergoing therapy, when the viral load falls there is an increase in Foxp3 positive cells and a representative example of the changes in Foxp3 cells during antiviral therapy is shown below (Data kindly provided by Dr L. Hibbert and Dr S. Al-Mazrouee)[207].

Figure 6.1.1 Changes in FoxP3 cells during interferon and ribavirin induced viral clearance

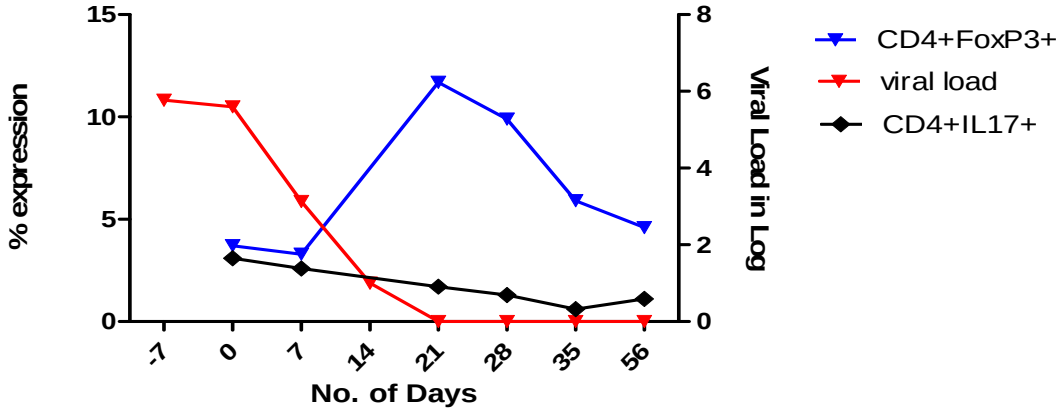


Figure 6.1.1 Samples were stained as described with anti-CD3, anti-CD4, anti-Foxp3 and anti-IL-17. The percentage of CD3+ cells that were either CD4+/Foxp3+ (▲) or CD4+/IL-17+ (■) and the viral load (▲) are shown at timepoints pre - and during IFN treatment [207].

Thus the role of T cells during persistence and elimination of HCV is unclear – in particular the importance of Foxp3 positive T cells is a matter of considerable debate with new data questioning the traditional model of Tregs facilitating viral persistence. One approach examining the role of Tregs in chronic HCV infection is to examine their presence following therapy in patients who do, or do not, undergo a virological relapse. If Tregs are important for viral persistence then we would predict that Foxp3 cells would increase in patients who relapse. If Tregs are important for viral clearance, as suggested by Godkin et al (2008) and our own data, then we would predict that Tregs would decrease in patients who relapse. The purpose of these experiments is to examine these different models.

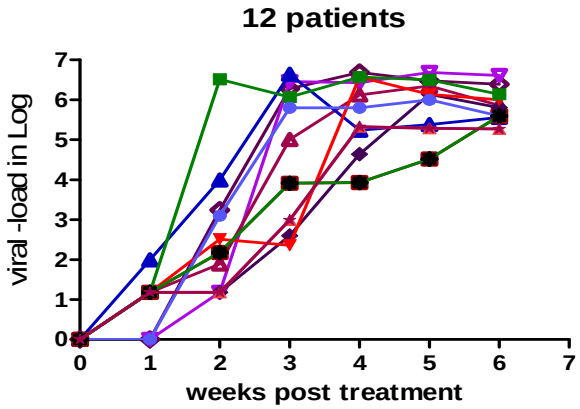
6.1.2 Results:Viral Kinetics of Relapse

Weekly plasma samples from 30 patients post-completion of treatment were monitored for HCV RNA. Twelve patients had a virological relapse and became HCV RNA positive 1-4 weeks post treatment. The kinetics of the return of viraemia in these patients is illustrated in Figure 6.1.2

Figure 6.1.2 The kinetics of HCV RNA Relapse

Twelve patients (of the 30 studied) who relapsed following a course of pegyalted interferon and ribavirin were studied in detail with HCV RNA evaluated weekly after therapy. The patients who relapsed did so at 2-6 weeks post-completion of treatment, apart from 1 patient who became HCV RNA positive after 9 weeks. Figure 6.1.2 shows the results in the majority of patients who relapsed early after discontinuation of therapy. In all patients relapse was very rapid occurring within 14 days of discontinuing therapy. No other patient from this cohort underwent relapse and therefore the return of viraemia after discontinuation of treatment is very rapid indeed.

Figure 6.1.2: The weekly viral loads of the patients who relapsed after cessation of IFN treatment. .



Viral loads, measured using a Roche Taqman automated assay approach are shown in 12 patients who relapsed following therapy with pegyalted interferon and ribavirin.

6.1.3 Results: Analysis of T cell subsets weekly for 6 weeks post treatment.

Of the 30 patients who were studied post treatment, the T cell subsets of 18 patients were followed weekly for six weeks. There was no discernable difference between relapsers and non-

relapsers therefore no further patients were investigated and the data presented here represents the preliminary studies from a series of experiments that refuted our primary hypothesis.

Of the 30 patients that were followed 12 relapsed and viable PBMCs were recovered from 18 patients of whom 4 relapsed. These data are shown here.

Figure 6.1.3 :CD4+ve/CD25+ in Relapsers

Viral load and % CD4+ve/CD25+ and CD8+ve T cells in patients treated with pegylated interferon and ribavirin who relapsed post treatment.

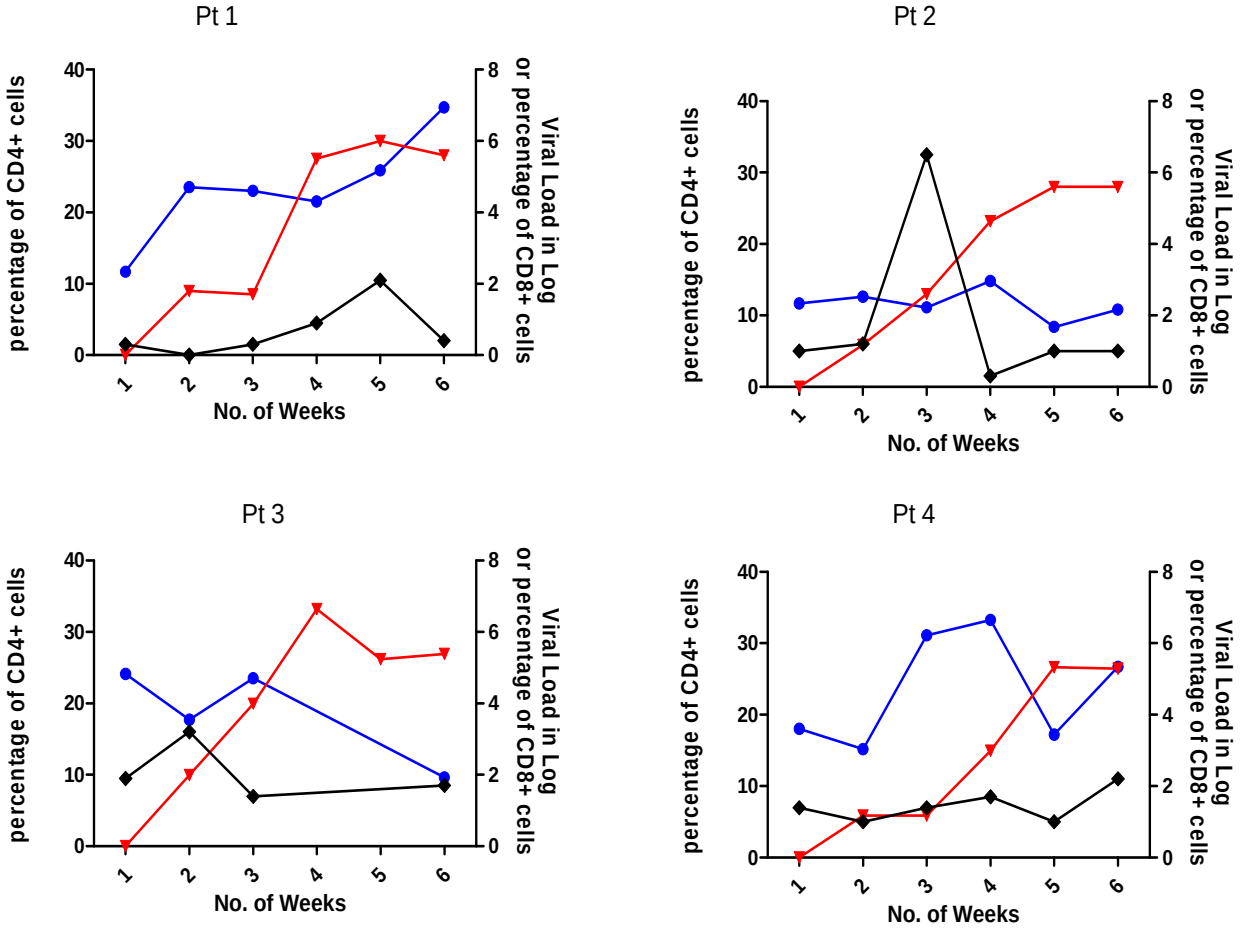


Figure 6.1.3:Samples were stained as described with anti-CD3, anti-CD4, anti-CD8 and anti-CD25. Percentage of CD3+ cells that were either CD4+/CD25+ (●) or CD8+/CD25+ (■) and the viral load (▲) are shown at various timepoints after cessation of IFN treatment.

Changes in CD25 expression can be seen after withdrawal of IFN/ribavirin treatment. In the relapsed patient 1, once the patient had stopped treatment, the percentage of CD4+/CD25+ T cells increased from 12% at week 0 to 23% at week one, steadily increasing to 35% at week six.

Patient 4 also a relapse patient had a similar profile, the CD4+/CD25+ T cells increased from 18% at week 0 straight after stopping treatment to 33% at week 4 and remained at 26% at week six as the virus reached its peak of 5 log viral load.

For the relapsed patient 2 after stopping treatment the CD4+/CD25+ increased from 12% at week zero and remained at 11% at week six as the virus reached its peak. In the relapsed patient 3 post treatment the CD4+/CD25+ T cells decreased, at week zero CD4+/CD25+ T cells were 24% of CD3+ cells but dropped to 9.6% at week six when the virus reached its peak of 5-6 log. Thus two patterns were seen an increase in CD4+/CD25+ T cells once the patient relapsed or a decrease in CD4+/CD25+ when the virus returns.

This is a small dataset and more patients will be needed to see if this dual response is consistent in all the patients who have relapsed or if there is another pattern of relapse. There were no major percentage changes in CD8+/CD25+ T cells in the four relapsed patients post treatment.

Changes in CD4+/CD25+ and CD8+/CD25+ were seen in patients who remained HCV RNA negative and responded to treatment .The results of those patients are as below in Figure 6.1.4

Figure 6.1.4: % CD4+ve/CD25+ and CD8+ve T cells in Responders

Viral load and % CD4+ve/CD25+ and CD8+ve/CD25+ T cells in patients treated with pegylated interferon and ribavirin who remained HCV RNA negative during follow-up.

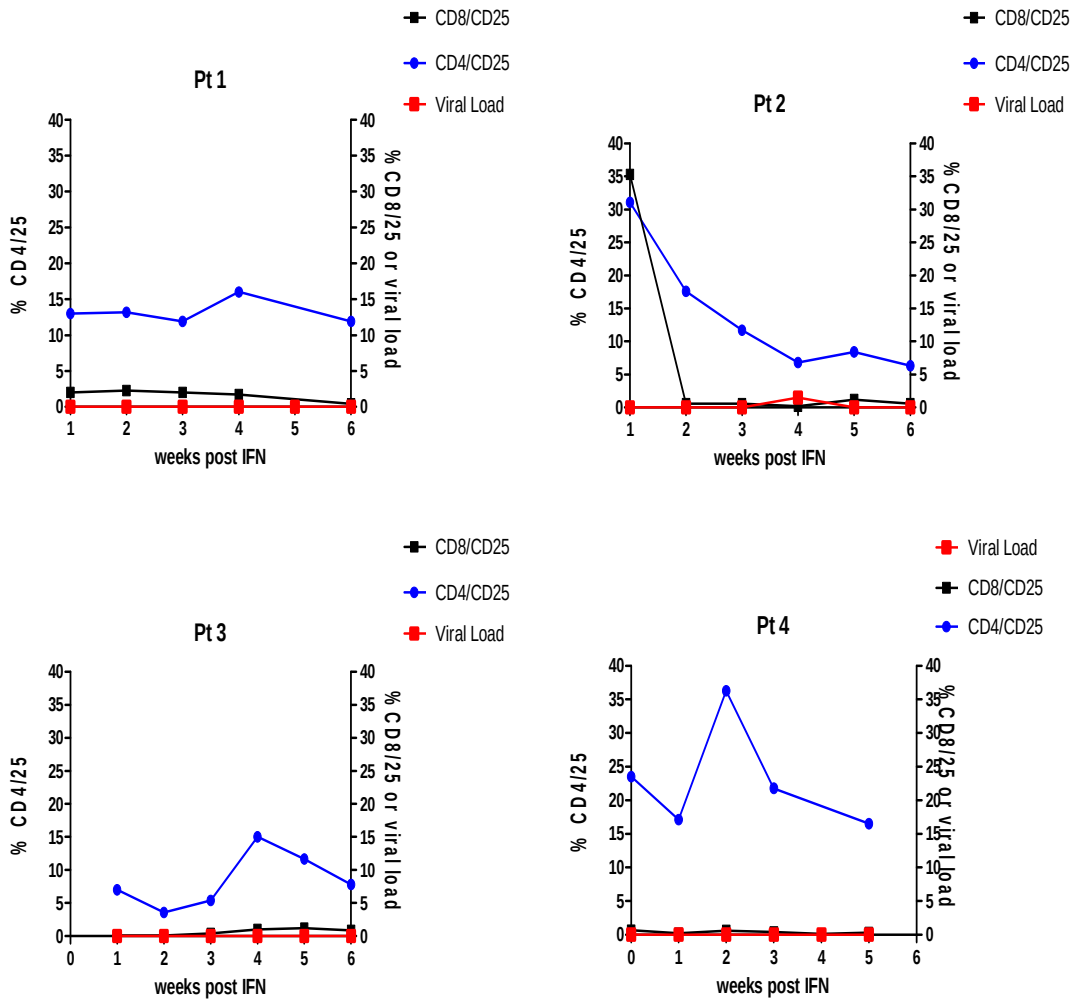


Figure 6.1.4: Samples were stained as described with anti-CD3, anti-CD4 , anti-CD8 and anti-CD25. Percentage of CD3+ cells that were either CD4+/CD25+ (●) or CD8+/CD25+ (■) or viral load (▲) are shown at various timepoints after cessation of IFN treatment.

Figure 6.1.4: % CD4+ve/CD25+ and CD8+ve T cells in Responders (Continue..)

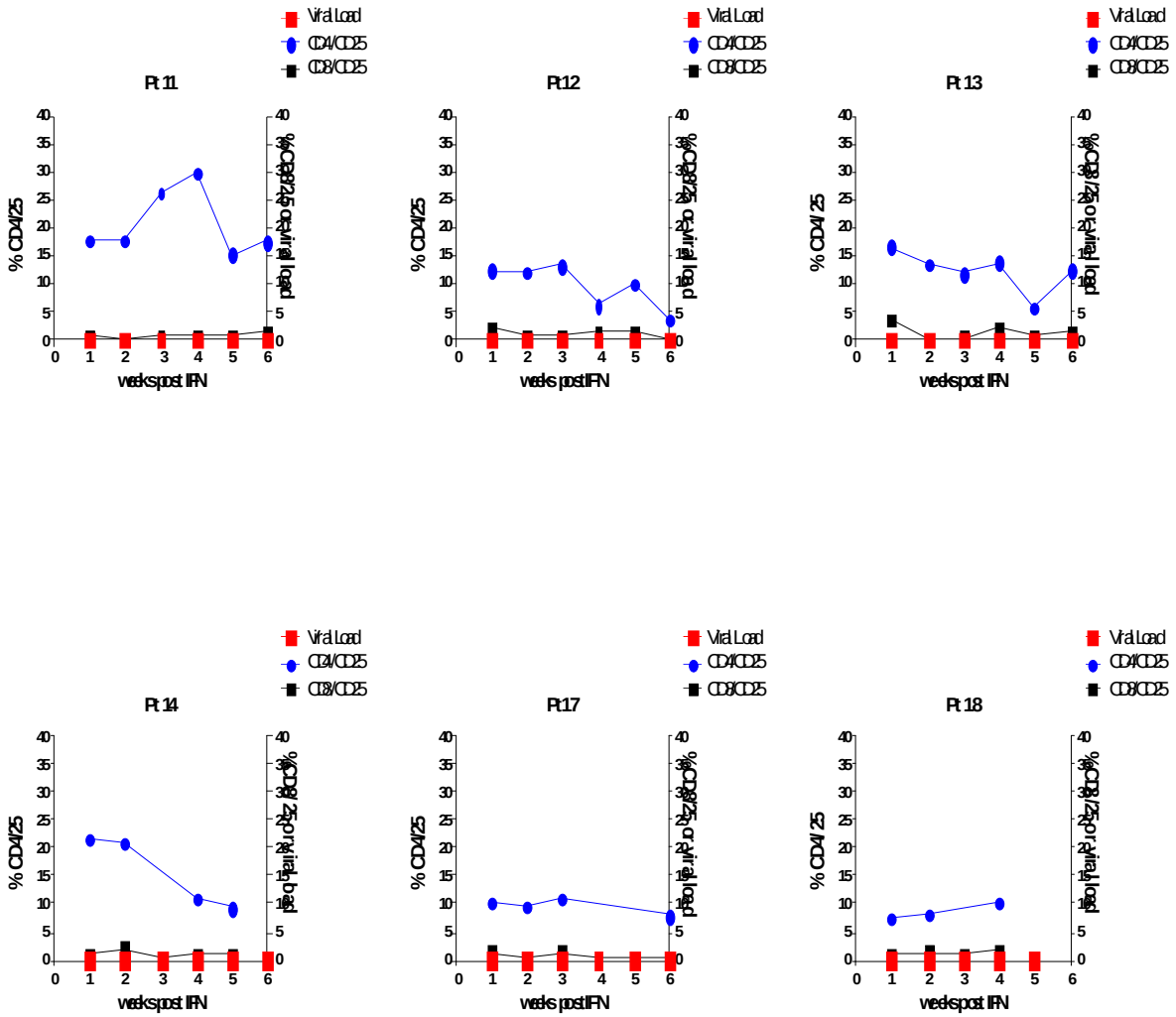


Figure 6.1.4: Samples were stained as described with anti-CD3, anti-CD4, anti-CD8 and anti-CD25. Percentage of CD3+ cells that were either CD4+/CD25+ (●) or CD8+/CD25+ (■) or viral load (▲) are shown at various timepoints after cessation of IFN treatment.

Three patterns of responses were seen in the patients who responded to treatment in the first six weeks post treatment. These patterns were a fluctuating level of CD4/CD25 during the first six weeks post treatment (patients 1, 3, 11, 13 and 18), a transient increase – patient 7, and 10 and a transient decline – patients 2, 4,6,9,12,14 and 17).

The above analysis suggests that the pattern of response may be complex and no predictive patterns may emerge but conducting the study in a larger population will be required to confirm this.

To determine whether changes in FoxP3 expressing T cells provided additional information when compared to CD25 expressing cells, we analysed cells by intracellular staining for FoxP3 and by staining for IL-17. Figure 6.1.5 shows the results

Figure 6.1.5 Changes in FoxP3 and IL-17 in Relapsers

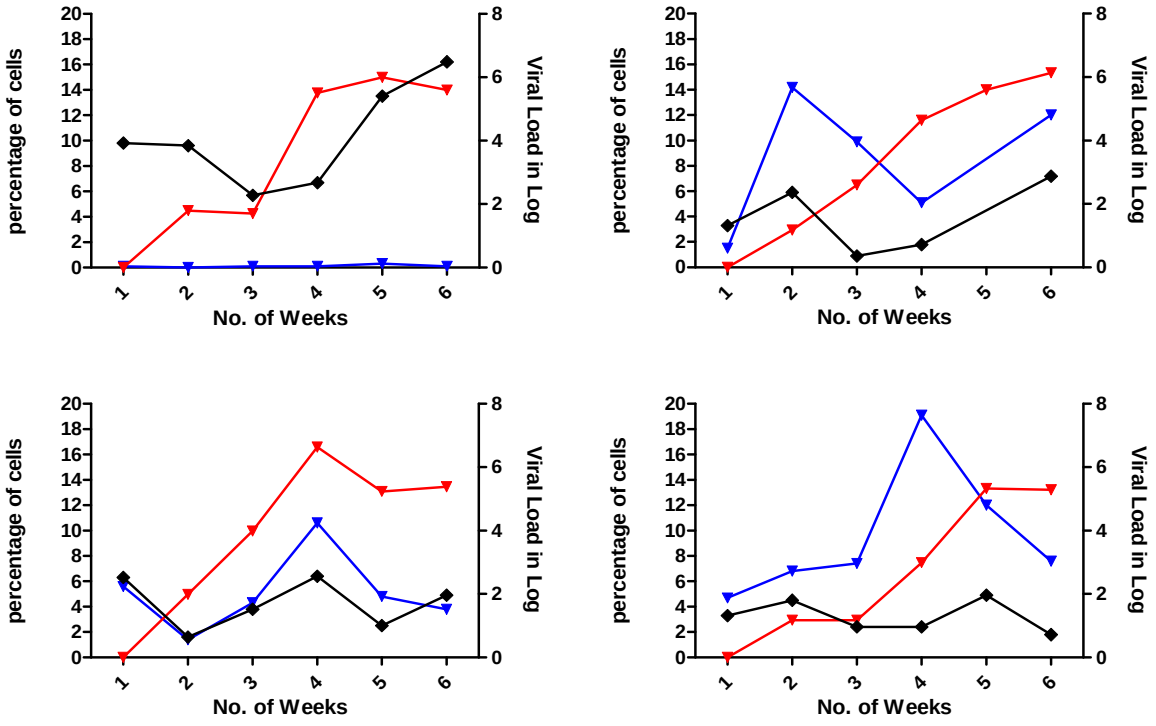


Figure 6.1.5: Samples were stained as described with aCD3, aCD4, aFoxp3 and aIL-17. Percentage of CD3+ cells that were either CD4+/Foxp3 (●) or CD4+/IL-17+ (■) or viral load (▲) are shown at various time points after cessation of IFN treatment.

The response differed in the four patients that relapsed post therapy with respect to changes in CD4/Foxp3 - Patient one showed a consistently low level of CD4/Foxp3 while the patient relapsed while patient 2 and 4 showed an increase of CD4/Foxp3 during relapse post treatment. Patient 3 showed a transient decline in CD4/FoxP3 after relapse post treatment.

Of the CD4/IL-17 two patterns are again seen:- in patient 1 and 2 there is an increase in CD4/IL-17 which remains consistently elevated as the patient relapses. Patient 3 and 4 shows a transient level of decline of CD4/IL-17 post treatment. This is a small dataset and more patients will be needed to see if this response is consistent in all the patients who have relapsed or if there is another pattern of relapse.

Figure 6.1.6 CD4/Foxp3-CD4/IL-17- in patients who responded to therapy

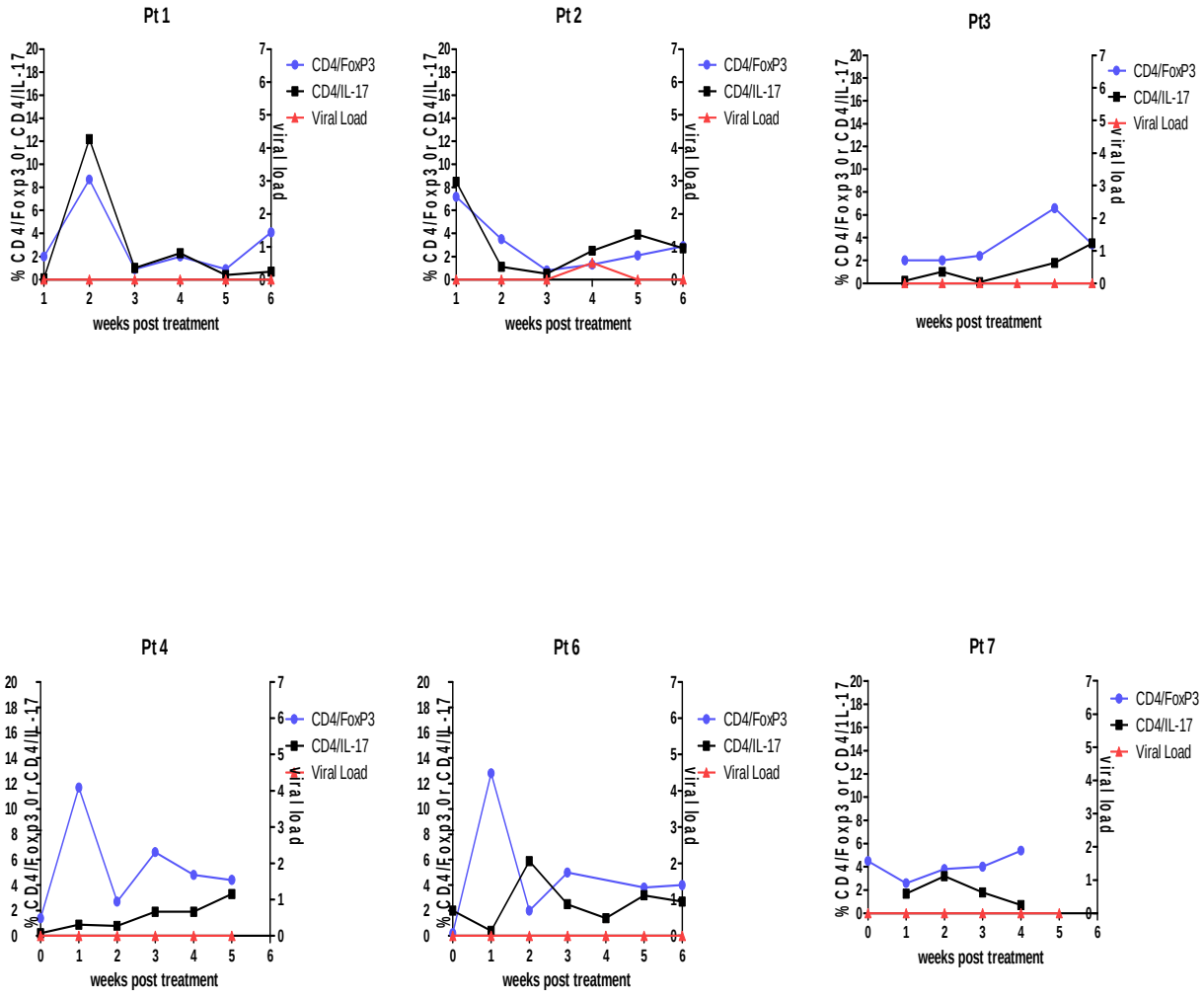


Figure 6.1.6 CD4/Foxp3-CD4/IL-17- in patients who responded to therapy (Continue..)

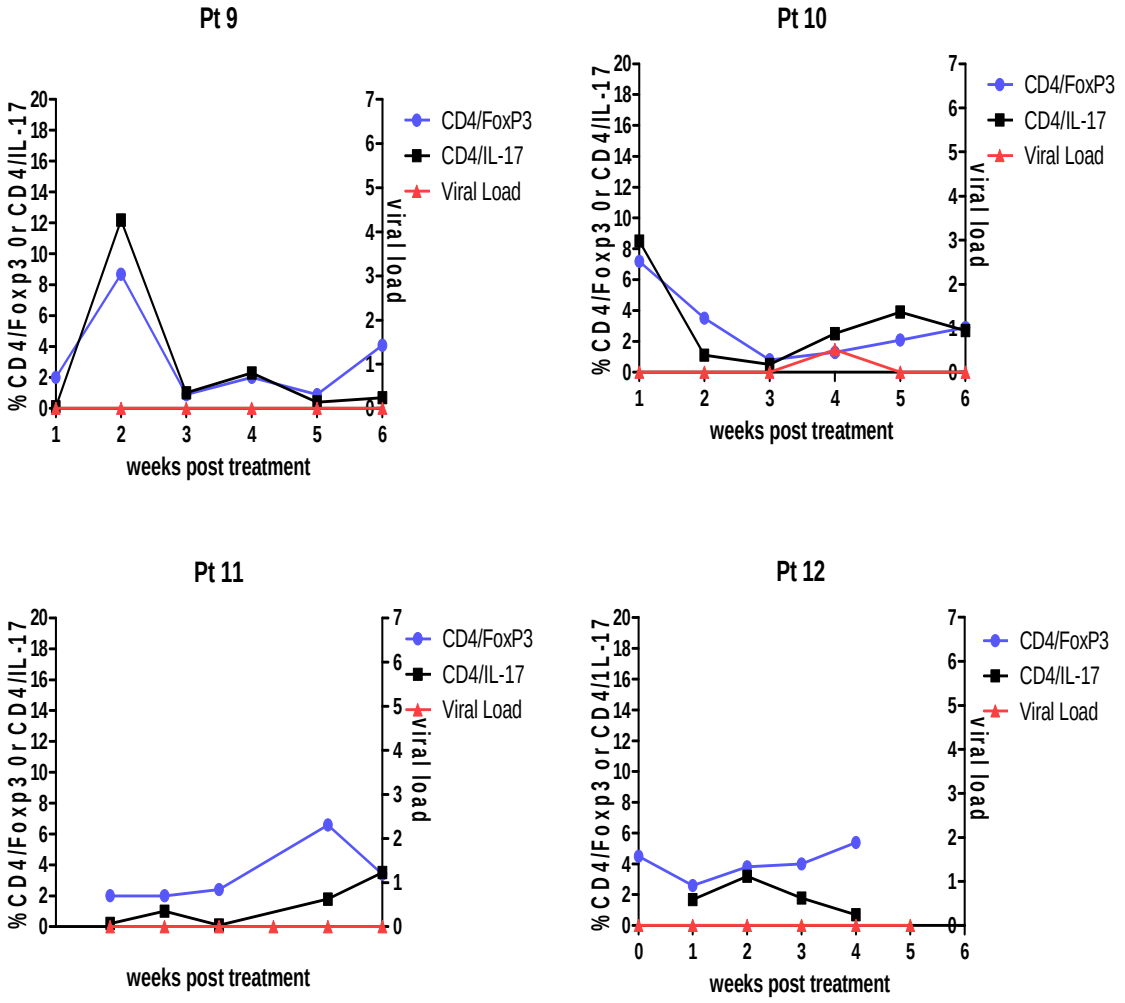


Figure 6.1.6: Samples were stained as described with aCD3, aCD4, aFoxp3 and aIL-17. Percentage of CD3+ cells that were either CD4+/Foxp3 (●) or CD4+/IL-17+ (■) or viral load (▲) are shown at various timepoints after cessation of IFN treatment.

Figure 6.1.6: CD4/Foxp3-CD4/IL-17- in patients who responded to therapy (Continue..)

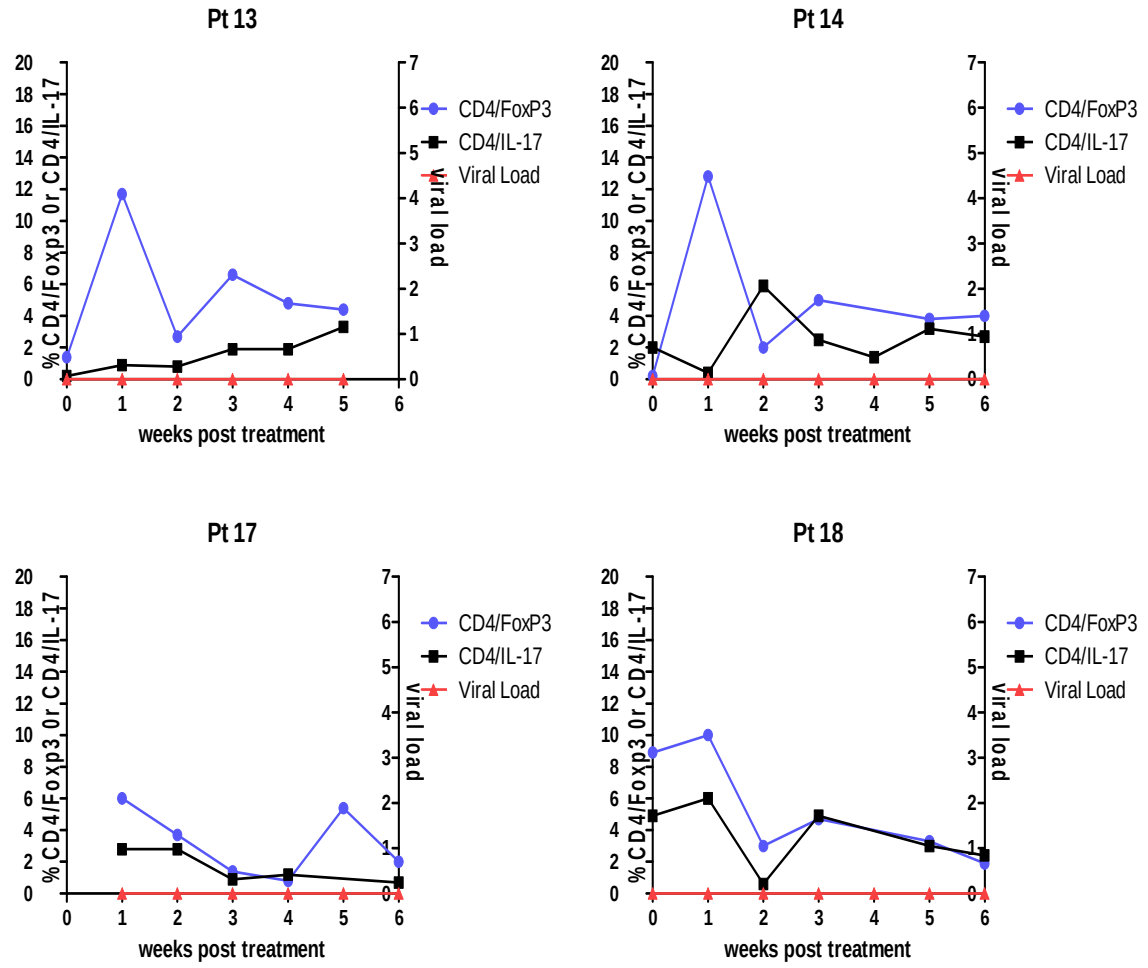


Figure 6.1.6: Samples were stained as described with aCD3, aCD4, aFoxp3 and aIL-17. Percentage of CD3+ cells that were either CD4+/Foxp3 (●) or CD4+/IL-17+ (■) or viral load (▲) are shown at various timepoints after cessation of IFN treatment.

The changes in CD4/Foxp3 in patients who have responded to therapy are shown above. Of the patients who remained virus negative six weeks post treatment two patterns were seen– a transient rise in CD4/Foxp3 (Patients 1,3,4,6,7,9,11,12,13 and 14) and a transient decline in CD4/Foxp3 (Patients 2,10,17 and 18).

Again two patterns of response were seen with the CD4/IL-17 –A transient increase in CD4/IL-17 was seen (patients 1,3,4,6,9,11,13 and 14) while a transient decline in CD4/IL-17 was also seen (Patients 7,10,12,17 and 18).

The small numbers preclude a formal analysis but our observations suggest that the changes in Foxp3 expressing cells may not be associated with virological outcome and more patients will be needed to determine whether or not this model is confirmed. CD69 might also be a marker for predicting response to therapy as the presence of anti-CD69 auto-antibodies are found to be associated with a poor response to interferon-α (IFN-α) therapy[208].

We therefore analysed changes in CD69 expression on lymphocytes in patients who did or did not relapse after therapy

Figure 6.1.7: Changes in CD4+/CD69+ & CD8+/CD69+ in Relapsers

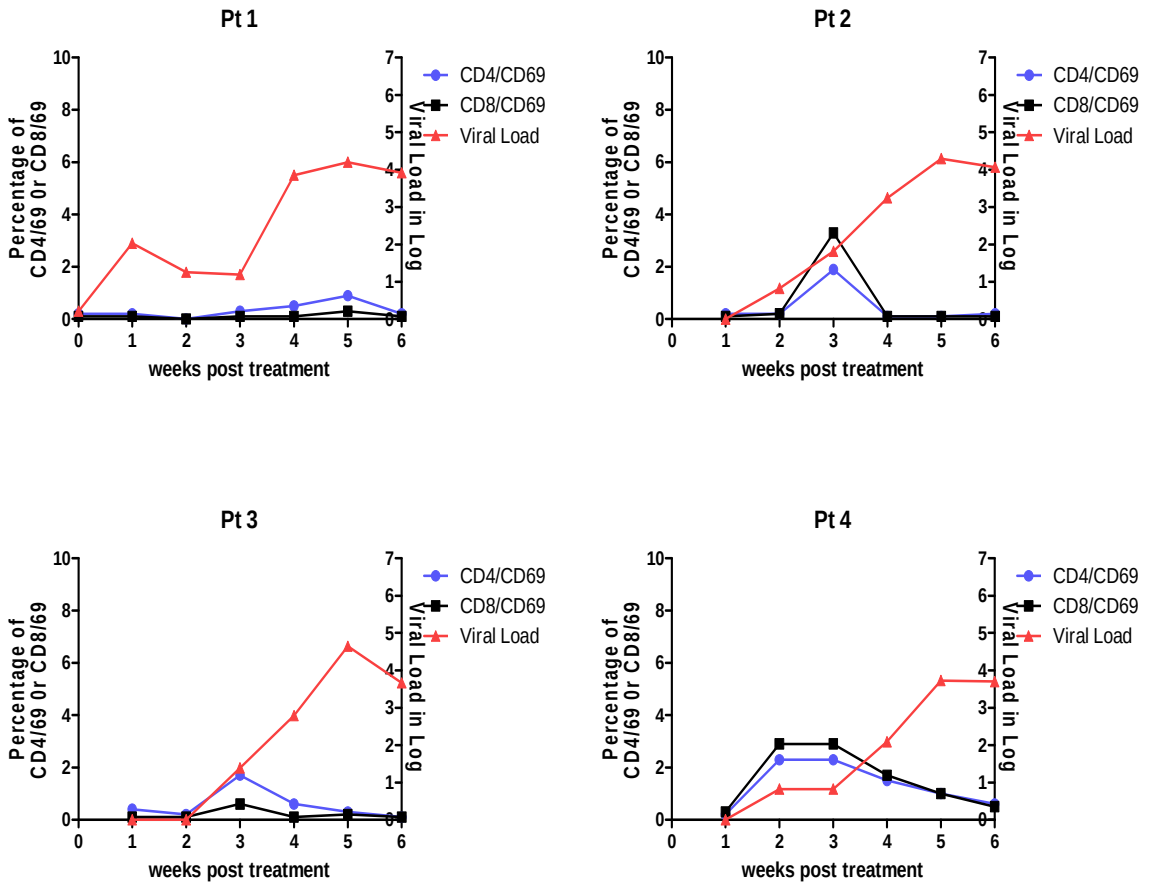


Figure 6.1.7: Samples were stained as described with aCD3, aCD4 and aCD69. Percentage of CD3+ cells that were either CD4+/CD69+ (●) or CD8+/CD69+ (■) or viral load (▲) are shown at various time points after cessation of IFN treatment).

Expression of CD69 is unchanged after removal of IFN/Ribavirin.There seems to be a transient fluctuation in CD69 in patient 2 and 4.There were no consistent changes in CD69 in any patient post therapy except for a small increase which was seen in one of the patients who relapsed.

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Figure 6.1.8: Changes in CD4+/CD69+ and CD8+/CD69+ in patients who responded to therapy.

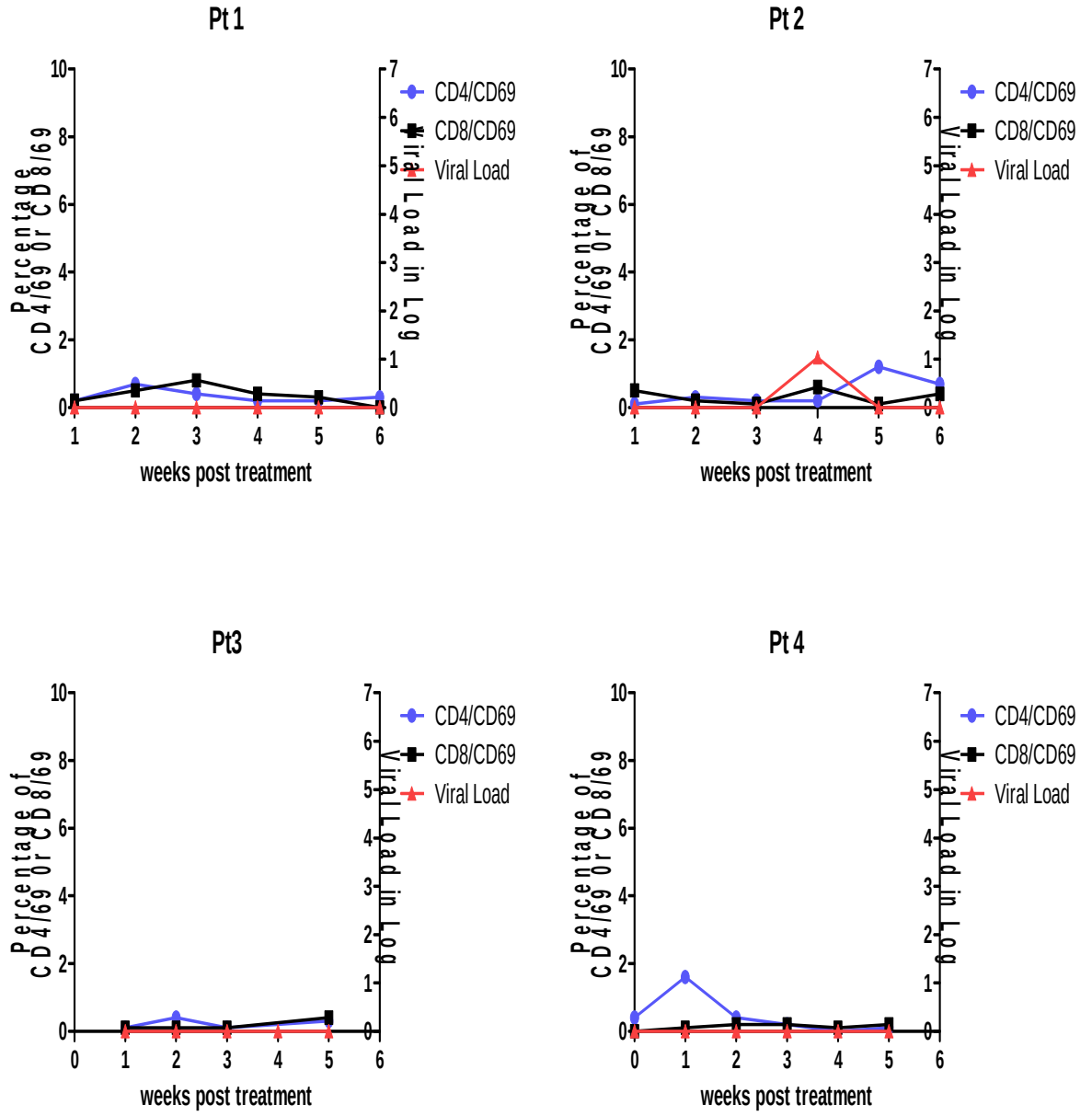


Figure 6.1.8: Samples were stained as described with aCD3, aCD4 and aCD69. Percentage of CD3+ cells that were CD4+/CD69+ (●) or CD8+/CD69+ (■) or viral load (▲) are shown at various time points after cessation of IFN treatment).

Figure 6.1.8: Changes in CD4+/CD69+ and CD8+/CD69+ in patients who responded to therapy (Continue..)

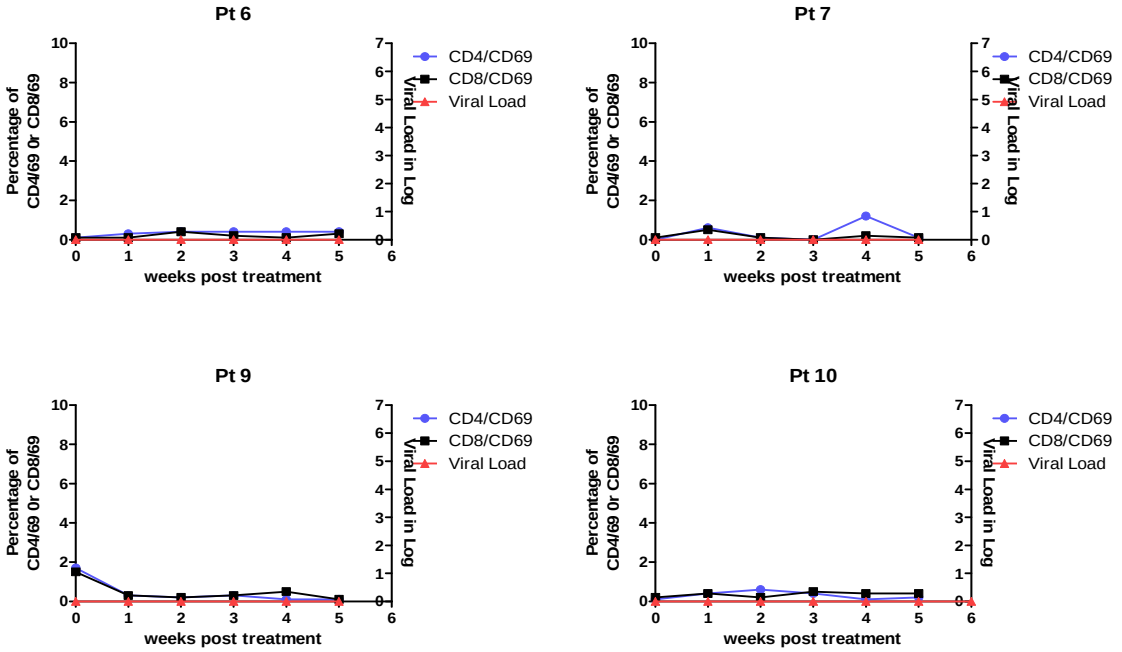


Figure 6.1.8: Samples were stained as described with aCD3, aCD4 and aCD69. Percentage of CD3+ cells that were CD4+/CD69+ (●) or CD8+/CD69+ (■) or viral load (▲) are shown at various time points after cessation of IFN treatment).

Figure 6.1.8 shows the changes in CD69 post therapy in patients who have responded to treatment. There were no changes seen in CD69 in any patient post therapy. There were no consistent changes in CD69 in any patient post therapy except for transient fluctuations of CD 69 which were seen in 2, 4,7,12, 14 and 17 in patients who responded to therapy.

The data presented above refute our hypothesis that changes in T cell sub populations may be associated with virological relapse as no consistent changes in T cell populations were observed in patients following antiviral therapy. However we did see fluctuations in some populations and to examine natural fluctuation in T cell populations we examined T cell changes in healthy volunteers. The data is shown below in Figure 6.1.9.

Figure 6.1.9:Changes in CD4/Foxp3 and CD4/IL-17 in Healthy Volunteers

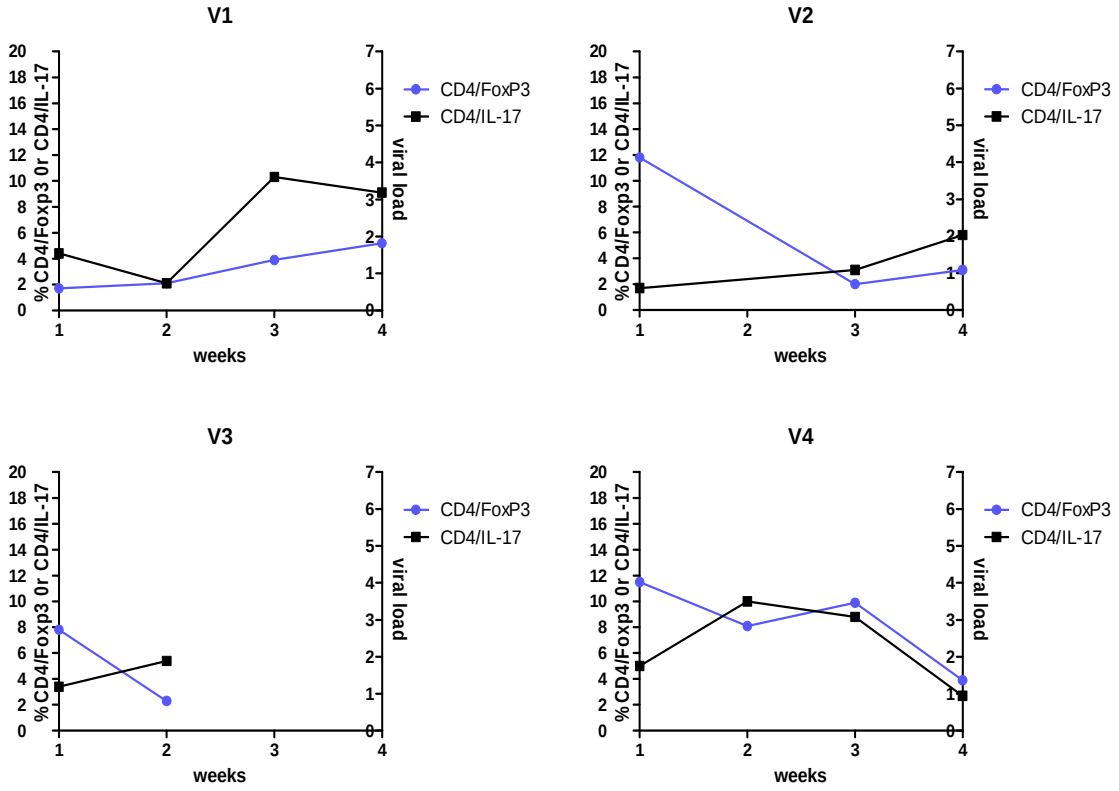


Figure 6.1.9: Samples were stained as described with aCD3, aCD4, aFoxp3 and aIL-17. Percentage of CD3+ cells that were either CD4+/Foxp3 (●) or CD4+/IL-17+ (■) are shown at various time points in healthy volunteers.

Four healthy volunteers were bled weekly for four consecutive weeks to look for changes in CD4/Foxp3 and CD4/IL-17.Fluctuating level of CD4/Foxp3 and CD4/IL-17 was seen in all three volunteers. The third volunteer was lost to follow up.

Figure 6.1.10:Changes in CD4+/CD25+ and CD8+/CD25+ in Healthy Volunteers

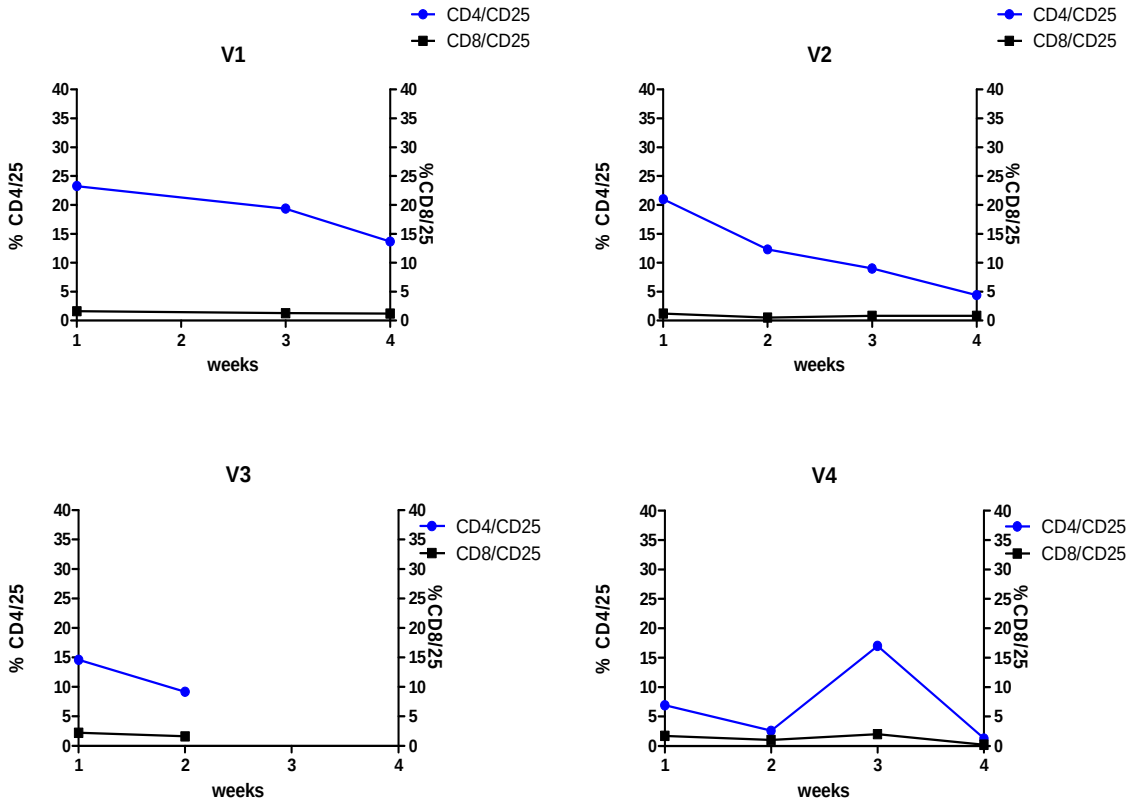


Figure 6.1.10: Samples were stained as described with aCD3, aCD4, aCD8 and aCD25. Percentage of CD3+ cells that were either CD4+/CD25+ (●) or CD8+/CD25+ (■) shown at various time points in healthy volunteers.

Of the four healthy volunteers studied over 4 weeks we studied changes in CD4/CD25 and CD8/CD25.Fluctuating level of CD4/CD25 and CD8/CD25 was seen in all three volunteers. The fourth volunteer was lost to follow up.

The studies in healthy volunteers indicate that changes in T cell populations occur over short periods of time (an alternative explanation is that the assay itself is highly variable) and taken together the data above suggest that a clear immunological signal of relapse is unlikely to be

detected without very large groups of patients where the 'noise' in the assay can be eliminated by statistical approaches. Given the low probability of identifying a clear population change we abandoned this line of work.

6.2: Analysis of viral quasispecies in patients who relapsed following pegylated interferon and ribavirin therapy.

6.2.1 Introduction

The Hepatitis C virus shows nucleotide sequence diversity throughout the viral genome. The HCV genome exhibits significant genetic heterogeneity with accumulation of mutations during viral replication, due to the lack of proof-reading ability of the RNA dependent RNA polymerase that results in the generation of closely related variants termed quasispecies [24]. The HCV genomic heterogeneity is not distributed evenly across the HCV genome. In particular, the untranslated region at 5' and 3' ends of the genome are the most conserved, whereas the hypervariable region 1(HVR1) located in the amino-terminus of the HCV envelope glycoprotein E2 is the most variable part of the HCV genome. There is strong evidence to suggest that the HVR1, encoding 27 amino acids (positions 1491 to 1571 on reference strain H77), is susceptible to immune pressure involving neutralising antibodies and allows the selection of escape mutants. Two other hypervariable regions have also been described, HVR2, 75 amino acids downstream of HVR1 in HCV genotype 1b infections [209], and HVR3 located between HVR1 and HVR2 in a group of patients infected with different genotypes [210]. In patients infected with HCV genotype 3a, HVR1 and two further regions, HVR495 and HVR575, have been described as the regions subject to variation within the single host [211] Finally, there is some evidence for variation in the course of infection associated with different HCV variants and in response to treatment with interferon (Kanai et al., 1992; Yoshioka et al., 1992; Takada et al., 1992a; Pozzato et al., 1991).This would indicate an important role for identification of genotype in the pretreatment assessment of patients with chronic hepatitis C [13]. A possible model that has been suggested is the continuous evolution of HCV and subsequent immune escape during chronic infection which would eventually lead to loss of immune control leading to the development and evolution of viral quasispecies[212]. In a study with 29 patients with chronic HCV genotype 1 infection pretreatment variables that might affect the outcome of treatment, genetic heterogeneity within the viral E1-E2 glycoprotein region (nucleotides 851 to 2280) was assessed by sequencing

10 to 15 quasispecies clones per patient from serum-derived PCR products[213] and suggested that increased heterogeneity was associated with treatment failure. Many other studies have looked at the genetic heterogeneity at baseline in an attempt to predict treatment outcome [24] and no clear consensus has emerged. By contrast large scale studies have not yet been conducted looking at the change of sequence post treatment in chronic HCV genotype 3 patients who have relapsed post treatment and changes in quasispecies in unsuccessful therapy have not been documented. In a study done looking retrospectively to investigate quasispecies evolution in a group of 10 chronically infected patients with genotype 3a, treated with pegylated α2a-Interferon and ribavirin it was found that there was a low level of quasispecies diversity in the group of patients who had attained a SVR pre treatment whereas in the patient who relapsed diversity was higher than in the SVR group but there was a decrease in diversity at the time of relapse [24]. This single small scale study has not been reproduced.

Re-emergence of HCV RNA during treatment suggests that the viral quasispecies detected are resistant to the treatment. Relapse post-therapy however may indicate that the viral quasispecies that emerge are sensitive to the treatment but have survived in a site inaccessible to the drug treatment. If this is the case then we would expect the viral quasispecies that emerge following relapse to be similar to those present prior to therapy. On the contrary if viral relapse is due to the emergence of a clone of interferon resistant HCV then we would expect the quasispecies that emerge post relapse to be very different from those seen prior to therapy. We used our cohort of patients where virological relapse had been detected very soon after therapy to address this hypothesis. To examine this the E2 region was used to monitor the quasispecies present in the serum prior to treatment and in those which emerged post treatment in HCV genotype 3 infected patients treated with pegylated interferon and ribavirin who relapsed post the completion of therapy. We chose the E2 region as it is highly divergent and therefore likely to provide a clear picture of the quasispecies diversity pre and post therapy.

6.2.2 Results; response to treatment

All 30 patients responded to treatment 29 by total loss of detectable HCV-RNA, 1 patient had a reduction of HCV-RNA which remained just detectable throughout treatment. Once treatment stopped 18 patients maintained a sustained viral response (SVR) but 11 relapsed, and the patient

who remained positive throughout had an increased viral load. The patients who relapsed did so at 2-6 weeks post-completion of treatment, apart from 1 patient who became HCV RNA positive after 9 weeks. (Data on those patients in whom weekly viral loads were obtained is shown in Section 6.1.1) The HCV subtype with which the patients who relapsed were infected, the duration of their treatment and the number of weeks post treatment they first had detectable HCV-RNA, are listed in Table 6.2.1

Table 6.2.1 Patients with Genotype 3 HCV who relapsed post treatment

Patient	Genotype	Ishak stage fibrosis	Treatment Length (weeks)	Weeks post-treatment became HCV-RNA positive
DT*	3a	5/6	24	week 4
FS*	3b	6 (no Bx)	24	week 4
ND	3a	6 (no Bx)	24	week 3
TA	3b	4/6	24	week 2
FK*	3a	5	24	week 4
BM*	3a	6 (no Bx)	38	week 3
MK*	3a	5/6	48	week 5
ZA	3a	5/6	48	week 2
MJ	3a	6 (no Bx)	48	week 4
MB	3b	6 (no Bx)	48	week 9
MA[a]	3b	6/6	24	week 2

*patients whose HCV-RNA was successfully PCR'd both pre and post treatment

[a]patient whose pre-treatment sample insufficient for extraction.

Results; Testing the reverse transcriptase (RT) and PCR conditions

Initially a 1-step reverse transcription and first round PCR was performed on both the pre- and post-treatment HCV RNA samples extracted from each patient, using the UTR and F4 primers and Superscript III Onestep RT-PCR system followed by a nested PCR using primers 745F and 2982R and the Expand High Fidelity PCR system (Humphries et al. 2009) (See Materials and

Methods for more details). In the samples that gave a product they were of the predicted size 2237bp, a representative gel is shown in Figure 6.2.1. .

The following Figure 6.2.1 RT PCR product from HCV RNA isolated from pre-treatment plasma samples of patients

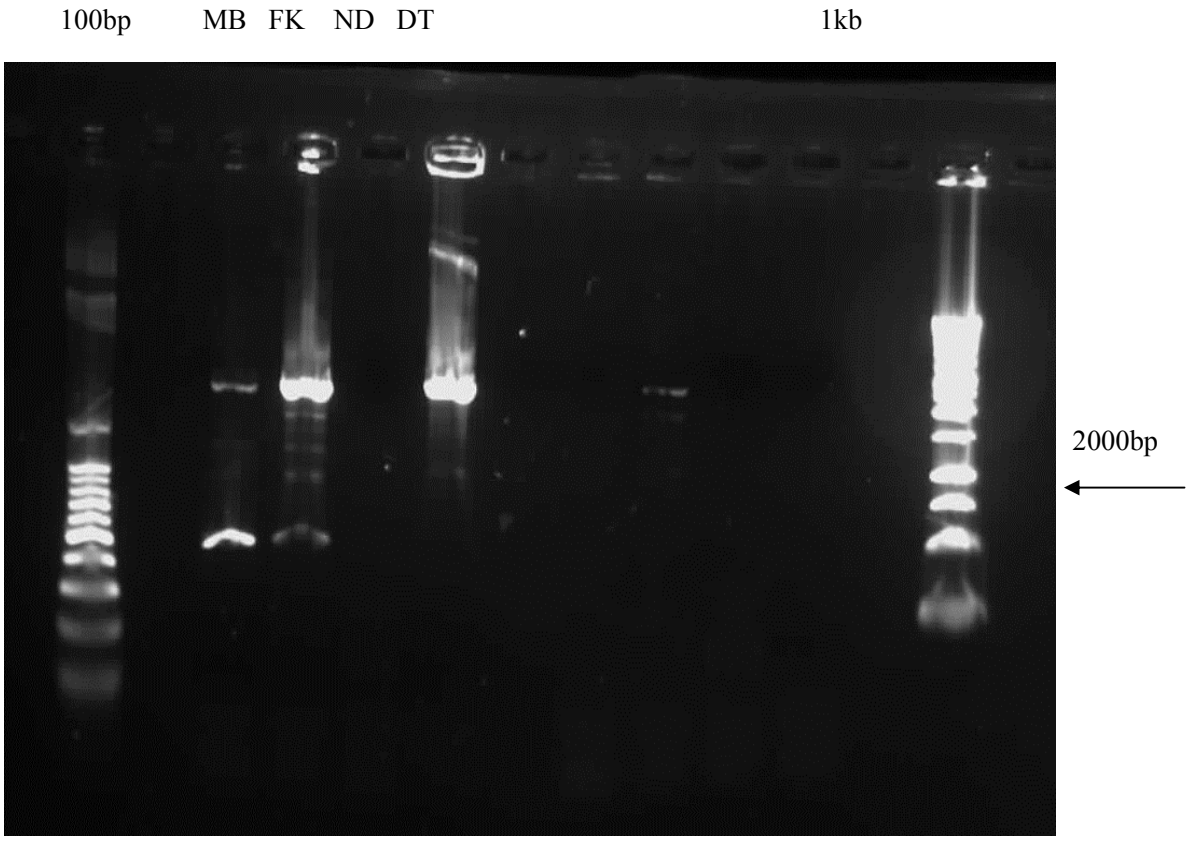
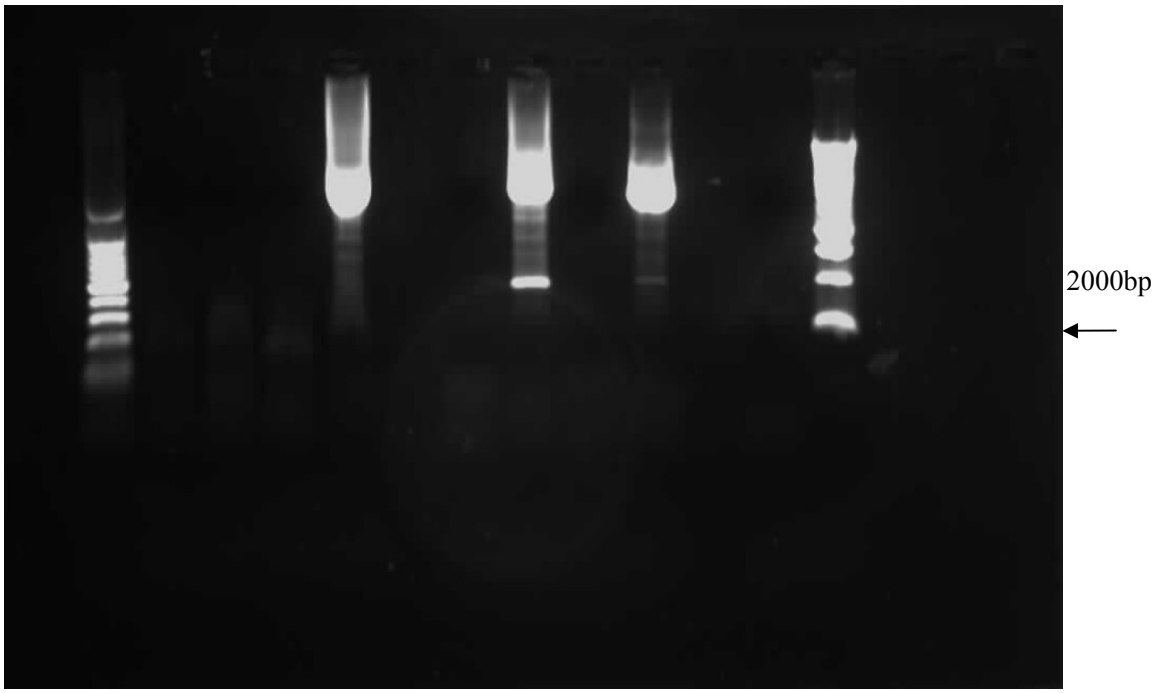


Figure 6.2.1 1% agarose gel of E2 PCR product from pre-treatment patient samples, patients MB, FK, ND and DT.

To be able to dilute the cDNA before PCR to obtain sequences from a single template, 2 different reverse transcriptase enzymes were compared in a 2 step RT PCR assay.

The same samples were reverse transcribed with either Finnzyme RT or Superscript III RT. Superscript III was found to be the more efficient enzyme as shown in Figure 6.2.2.

Figure 6.2.2 Comparison of E2 PCR product using either Finnzyme RT or Superscript III RT



6.2.4 Analysis of HCV Quasispecies

PCR product from bp745 to bp2982 (patient MK bp2788) (numbering relative to reference sequence AF009606) from both the pre- and post-treatment samples was obtained in 5 of the patients who relapsed, in the remaining patients either both the pre- and the post samples gave no product (n=4 patients) or only the pre-treatment sample gave a product (n=2 patients). Attempts were made to obtain PCR products within the E2 region from these patients with other combinations of primers but they were not successful.

These experiments were completed in conjunction with Dr.Jenny Waters .PCR products generated were cloned into pGEM-T vectors (work performed by Dr J. Waters) and 20 clones were used for sequencing in both directions over the E2 region (sequencing kindly performed by the Genome Centre at QMUL from cloned samples provided by Dr Waters from my samples and PCR products).

The sequences were trimmed to start at bp1491 (numbering relative to reference sequence AF009606) the beginning of the E2 region. The sequences were between 950 and 1050bp long and included the HVR1 region, the regions described as hypervariable in genotype 1 viruses HVR2 and HVR3, and those identified in genotype 3a viruses HVR 495 and HVR 575 [211].

To confirm that the sequences obtained for each patient all did come from that patient and there was no cross contamination between samples all of the sequences were aligned together. The sequences derived from each patient segregated by patient on the resulting guide tree (Figure 4.2.3).

In addition each of the patients had a distinct number of bases inserted in the region around HVR 575 when compared to the AF009606 reference sequence. For example all of patient DT sequences had a 24bp insert between nt 2062 & nt2063 and a 12bp insert between nt 2065 & nt2066 with respect to the reference sequence AF009606 whereas all of patient FS had 3 bases between nt2054 & nt2055, 6 bases between nt2062 & nt 2064, 6 bases between nt2064 & nt2065 and 3 bases between nt2081 & nt2082.

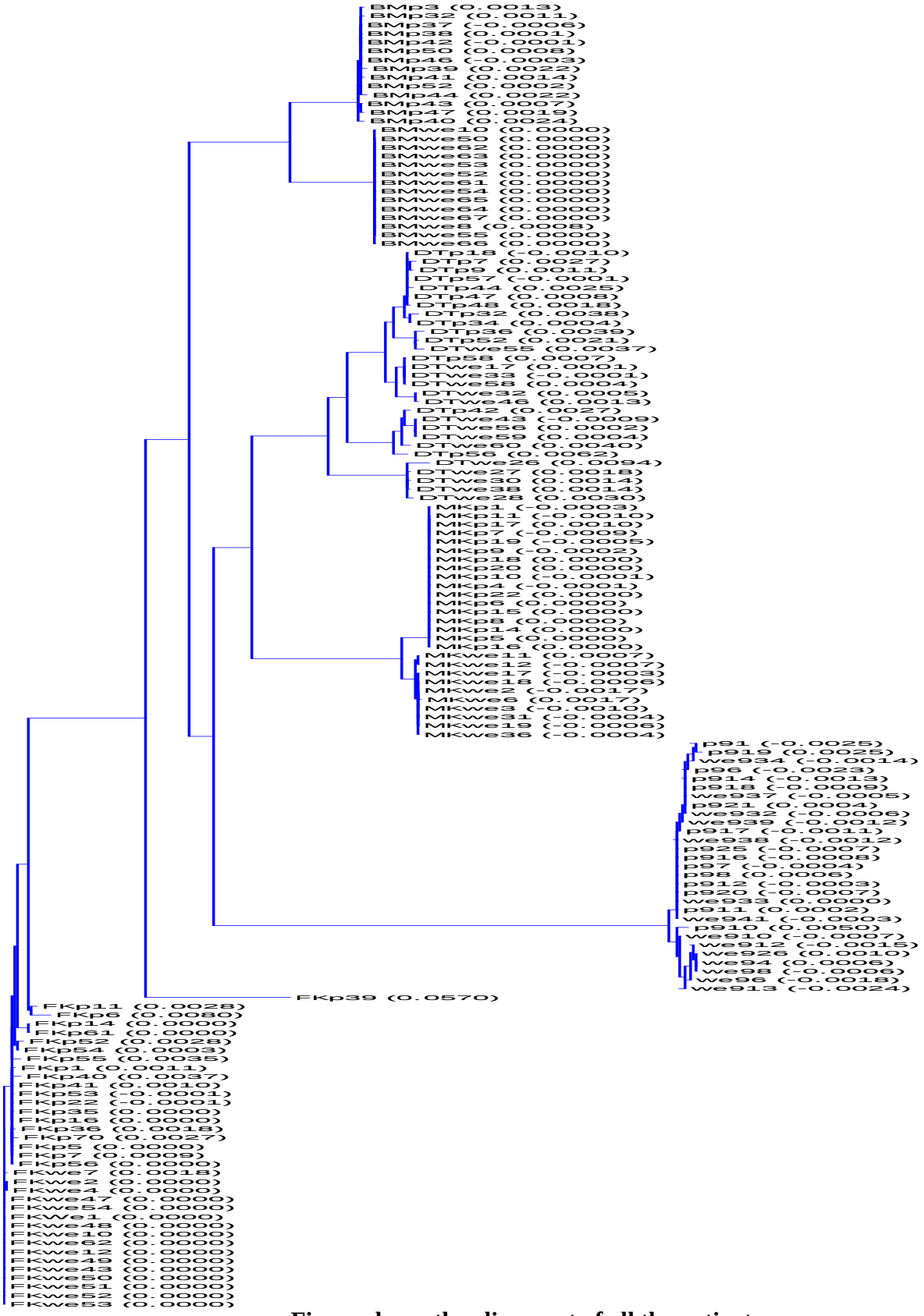


Figure shows the alignment of all the patient sequences

The guide tree was constructed using a modified Clustal W algorithm and multiple pairwise alignments within the Vector Nti program (Invitrogen), the patients are identified by their initials the samples without initials are from patient FS [‘p’ are pre- and ‘we’ post-treatment clones]

In two of the patients who relapsed, DT & FS, multiple HCV quasispecies re-emerged once treatment stopped the majority of which were represented in the pre-treatment sample (Figure 6.2.4). Patient DT had no dominant sequence either pre-treatment or post-treatment and had multiple differences in sequence within the HVR1 region [between nucleotides (nt) 1491-1571(all numbers are in relation to the ref. seq. AF009606)]. Patient FS had two groups of sequences both pre and post treatment. In the HVR1 region, there was just 1 base change in any of the sequences the majority of changes were outside of this region.

Three of the patients studied had changes in the E2 region which resulted in the emergence of new quasispecies post-treatment which had not been present in the pre-treatment sequences (Figure 6.2.5).

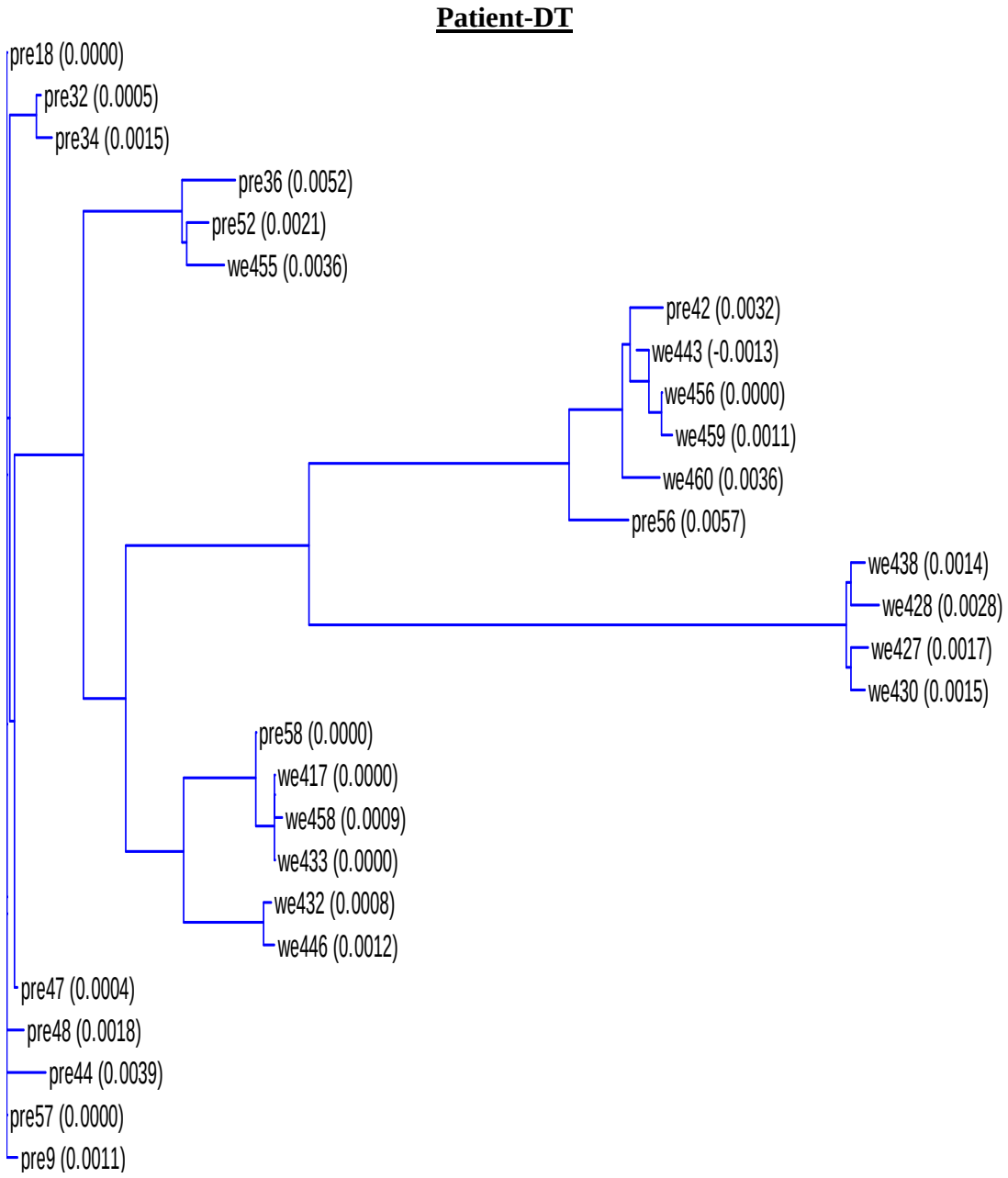
Patient BM became HCV-RNA positive at week 3 after ceasing treatment, the week 4 sample was processed and sequenced as there was insufficient of week 3. A single dominant quasispecies emerged post treatment, which when compared with the pre-treatment sequences had 19bp differences in the HVR1 region and multiple further base changes outside of that region.

In patient FK post treatment a dominant group of sequences emerged which was not represented in the pre-treatment sample. All of the post treatment sequences had 3 base changes which distinguished them from the pre treatment samples 1 in the HVR1 region and 2 in the HVR 575 region. In addition other base changes occurred throughout the sequences but not in all of the pre or post treatment samples.

In the pre-treatment samples of patient MK there was a single dominant sequence which differed from the sequences found post-treatment by 10 bases in the HVR1 region, 1 base in the HVR 575 region and 5 bases in the other parts of the E2 sequence.

Figure 6.2.3 Guide trees for two of the patients who had a mix of quasispecies pre-treatment which re-emerged post-treatment.

Guide trees were constructed using a modified Clustal W algorithm and multiple pairwise alignments within the Vector Nti program (Invitrogen) [‘p’ are pre- and ‘we’ post-treatment clones]



Patient FS

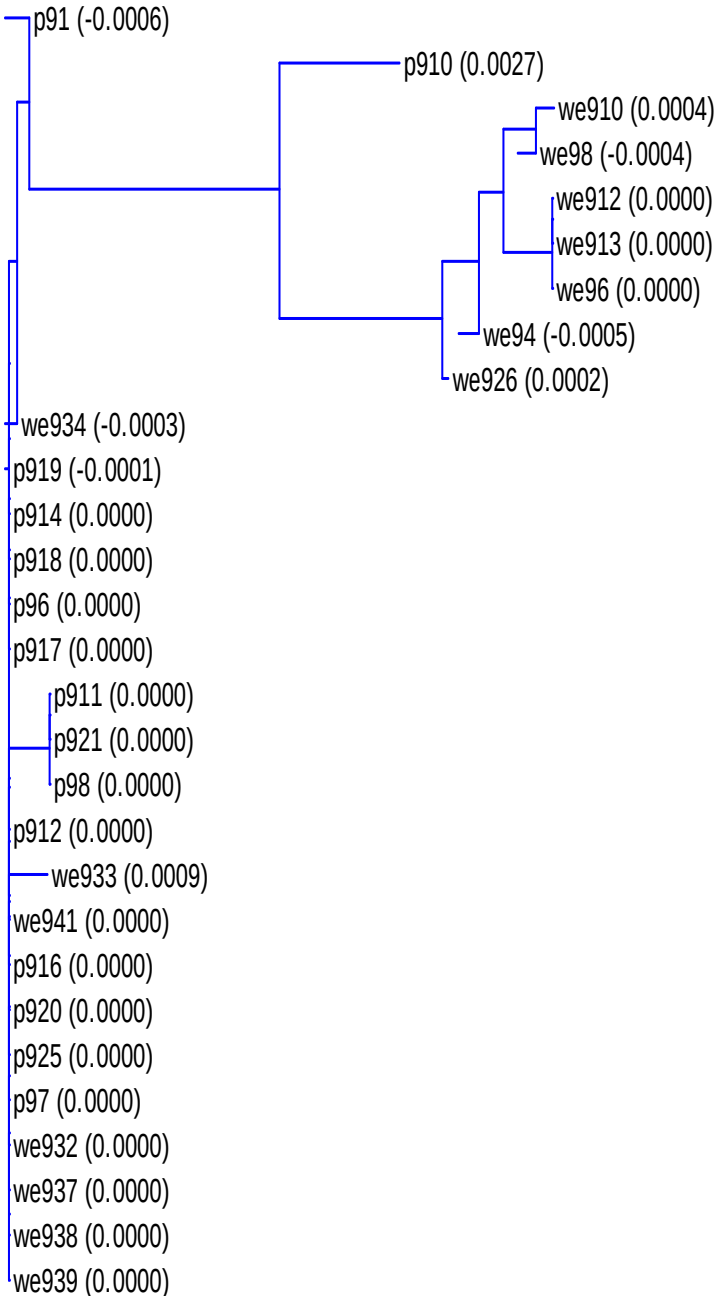
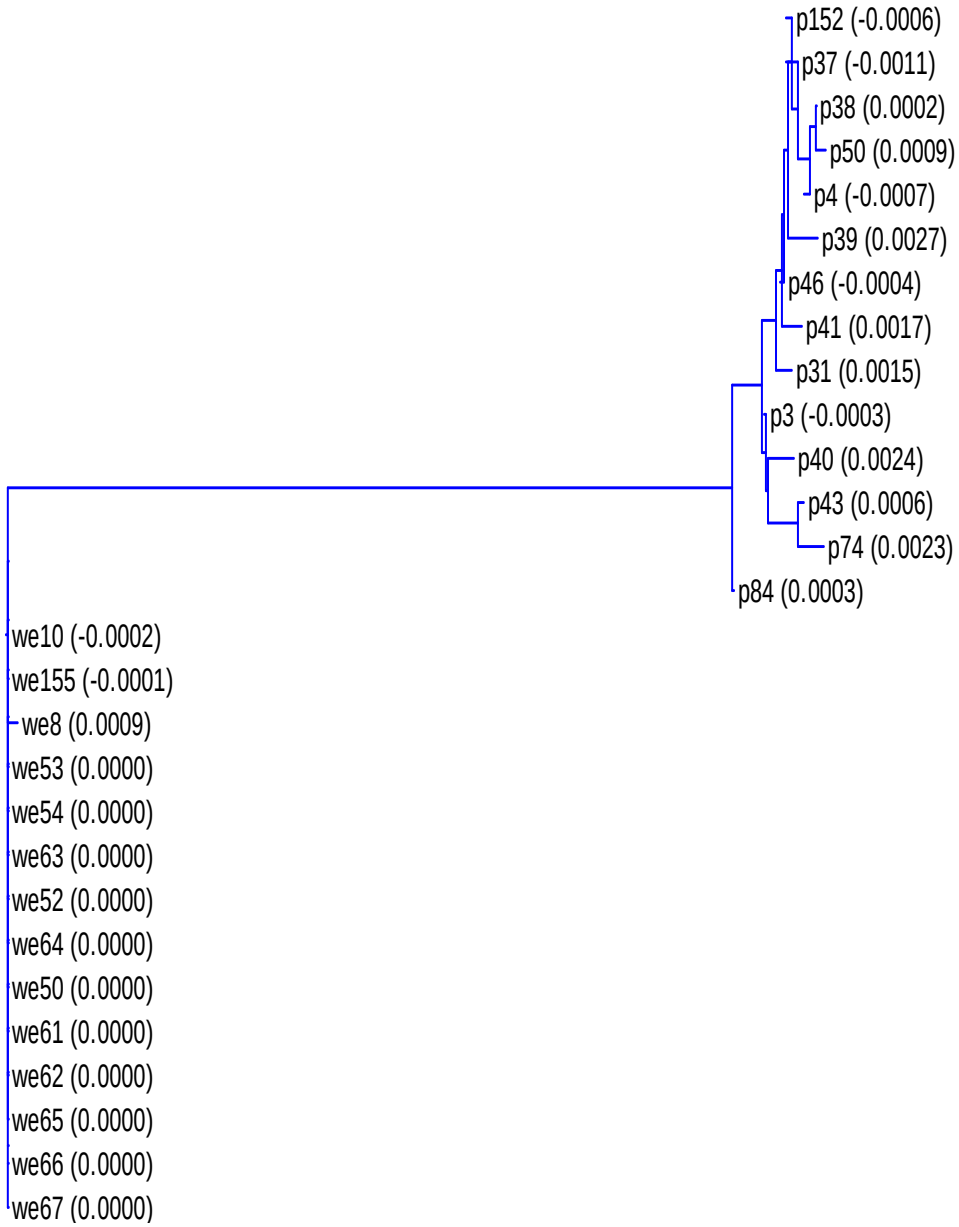

p91 (-0.0006)
p910 (0.0027)
we910 (0.0004)
we98 (-0.0004)
we912 (0.0000)
we913 (0.0000)
we96 (0.0000)
we94 (-0.0005)
we926 (0.0002)
we934 (-0.0003)
p919 (-0.0001)
p914 (0.0000)
p918 (0.0000)
p96 (0.0000)
p917 (0.0000)
p911 (0.0000)
p921 (0.0000)
p98 (0.0000)
p912 (0.0000)
we933 (0.0009)
we941 (0.0000)
p916 (0.0000)
p920 (0.0000)
p925 (0.0000)
p97 (0.0000)
we932 (0.0000)
we937 (0.0000)
we938 (0.0000)
we939 (0.0000)

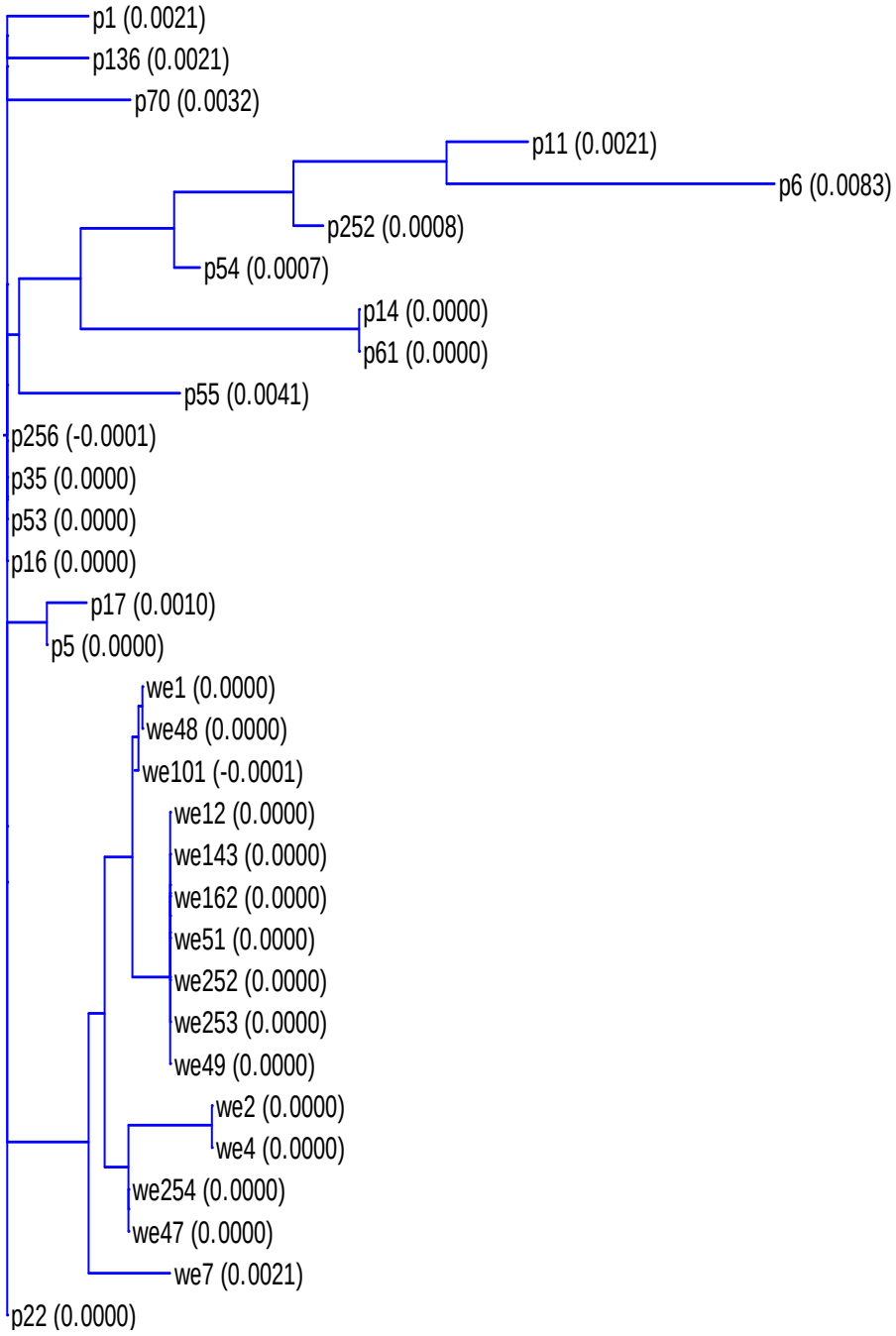
Figure 6.2.4 Guide trees of the HCV sequences from three patients who had different quasispecies pre- and post treatment treatment

(Guide trees were constructed using a modified Clustal W algorithm and multiple pairwise alignments within the Vector Nti program (Invitrogen) [‘p’ are pre- and ‘we’ post-treatment clones]

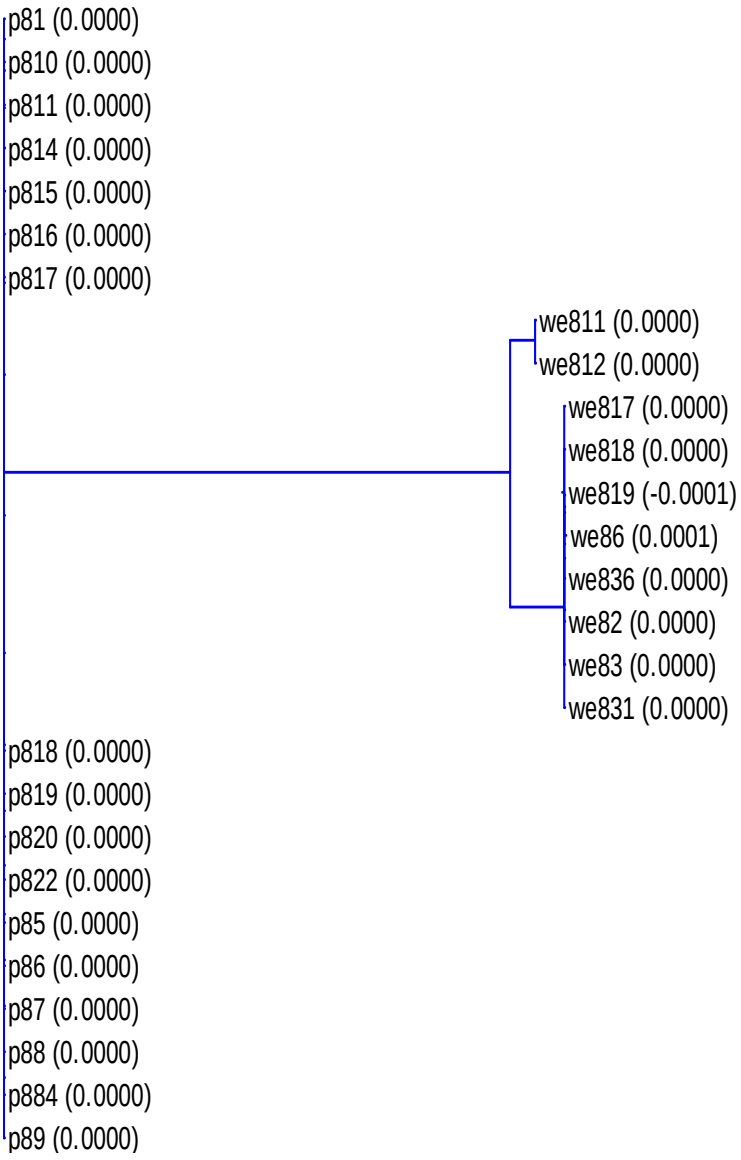
Patient BM



Patient FK


p1 (0.0021)
p136 (0.0021)
p70 (0.0032)
p11 (0.0021)
p6 (0.0083)
p252 (0.0008)
p54 (0.0007)
p14 (0.0000)
p61 (0.0000)
p55 (0.0041)
p256 (-0.0001)
p35 (0.0000)
p53 (0.0000)
p16 (0.0000)
p17 (0.0010)
p5 (0.0000)
we1 (0.0000)
we48 (0.0000)
we101 (-0.0001)
we12 (0.0000)
we143 (0.0000)
we162 (0.0000)
we51 (0.0000)
we252 (0.0000)
we253 (0.0000)
we49 (0.0000)
we2 (0.0000)
we4 (0.0000)
we254 (0.0000)
we47 (0.0000)
we7 (0.0021)
p22 (0.0000)

Patient MK


p81 (0.0000)
p810 (0.0000)
p811 (0.0000)
p814 (0.0000)
p815 (0.0000)
p816 (0.0000)
p817 (0.0000)
we811 (0.0000)
we812 (0.0000)
we817 (0.0000)
we818 (0.0000)
we819 (-0.0001)
we86 (0.0001)
we836 (0.0000)
we82 (0.0000)
we83 (0.0000)
we831 (0.0000)
p818 (0.0000)
p819 (0.0000)
p820 (0.0000)
p822 (0.0000)
p85 (0.0000)
p86 (0.0000)
p87 (0.0000)
p88 (0.0000)
p884 (0.0000)
p89 (0.0000)

Discussion

30 chronically HCV genotype 3 infected patients with advanced fibrosis (fibrosis score >F3/6) were treated with pegylated interferon and ribavirin for 24-48 weeks to determine the effectiveness of this regime in these patients. All of the patients responded with loss of virus on treatment of these 18 had a sustained viral response (SVR) and 12 patients relapsed post-treatment, within 4-6 weeks of the end of treatment. The SVR was 60% in these patients and those who relapsed were studied further in an attempt to define immunological and virological profiles associated with relapse.

Regulatory T cells are divided into natural $CD4^+$ $CD25^+$ Treg cell population and a population of induced or adaptive Treg cells. The major function of the natural or induced Tregs is the suppression of immune responses to self or foreign antigens. Studies done in mice with herpes simplex virus infection (HSV) showed that if mice who were suffering from blinding keratitis were depleted from Treg cells they showed a more severe eye lesion in the eye when depleted of nTreg cells [29], perhaps suggesting that Tregs reduce virally induced immunopathology.

In our preliminary studies Treg cells appeared to be associated with viral clearance and we therefore examined changes in Tregs following viral clearance or relapse. During these studies we also took the opportunity to examine other T cell markers including the activation marker CD69.

When looking at changes post treatment in CD4/CD25 cells in patients who have relapsed post treatment our data shows that there were two patterns i.e. an increase in CD4/CD25 once the patient relapses and the a CD4/CD25 decrease with return of viraemia. The pattern remains unchanged for CD8/CD25 post treatment.

When looking at changes in CD4/CD25 in patients who responded to therapy -three patterns of response were seen. These patterns were a fluctuating level of CD4/CD25, a transient increase in CD4/CD25 and a transient decline in CD4/CD25 during the first six weeks post treatment.These data suggests that the pattern of response may be complex and no predictive patterns emerged. It is unclear whether these changes simply reflect background fluctuation following interferon withdrawal and since no clear pattern emerged this approach was not pursued.

A similar pattern was seen with the other T cell markers studied and as no clear lines for further investigation presented themselves we did not pursue further studies in these areas.

Of the 12 patients who had relapsed post treatment 7 patients were genotype 3a and four of the patients were genotype 3b with one of the patients having a mixed genotype 3a/3b.Pre treatment and post treatment serum samples were available for all the 12 patients. The pre treatment sample was collected before the start of treatment and the post treatment samples were collected at end of treatment to six weeks post treatment to look for relapse. All of the patients relapsed very quickly after treatment was stopped. Of the 12 patients pre treatment PCR product was successfully generated for 7 patients and for 6 patients post treatment. I linked in with an experienced post doc Dr. Waters who helped in analyzing the sequence data and in constructing the phylogenetic trees. We were successfully able to generate PCR products from five of the patient samples to look for changes pre and post treatment. As part of the analysis of these patients HCV RNA was extracted from the pre-treatment sample and the first HCV-RNA positive post- treatment sample and the quasispecies diversity was assessed in 5 patients by sequencing the E2 region, including the HVR1, 2 &3 regions and the HVR 495 and HVR 575 region, of 12-15 clones in both pre- and post-treatment samples.

Construction of phylogenetic trees showed that in 2 patients the quasispecies that emerged post treatment were represented in the pre-treatment sample but in 3 of these patients new quasispecies not present in the pre-treatment samples emerged suggestive of a bottleneck in replication. In these 3 patients the change in quasispecies supports the idea that relapse was due either to the emergence of an interferon resistant virus or to sequestration of virus in a protected site. There are a number of possible explanations for these results including sequestration of virus in a sanctuary site, relapse from an area of the liver where the drugs have poor entry, perhaps because of fibrosis or rapid mutation of the virus.

However given the heterogeneity of the response firm conclusions can not be drawn at this stage and further studies will be required to analyse viral changes in more subjects. Nevertheless these interesting observations suggest that there may be two patterns of relapse following withdrawal

of antiviral therapy – one consisting of the development of novel viral quasispecies linked to an unusual pre-treatment variant (perhaps indicating the emergence of interferon resistant viral strains) and one pattern consisting of re-emergence of all of the pre-treatment quasispecies, (perhaps indicating relapse from a sanctuary site).
Further studies in this area would be of great interest and it would be interesting to evaluate the response to re-treatment with interferon in patients with these different patterns of response – if our hypothesis is correct then patients with a 'novel quasispecies pattern' should not respond to re-challenge with interferon as the virus should be resistant whereas patients with a re-appearance of pre-treatment quasispecies should show a response to re-challenge.

These pilot exploratory studies are of interest but the immune system studies are unlikely to be productive but studies on the virus and viral quasispecies development maybe worthwhile.

Chapter 7

DISCUSSION AND RECOMMENDATIONS

7.1 DISCUSSION AND RECOMMENDATIONS

This thesis examines the clinical, virological and immunological factors associated with treatment failure in patients with Genotype 3 chronic HCV infection. At the outset we hypothesised that patients from the Indian sub-continent (South Asians) would respond differently to therapy with pegylated interferon and ribavirin than Caucasians and we hypothesised that an analysis of viral and host factors underlying the difference would reveal new insights into the virology of Genotype 3 HCV infection. We further speculated that increasing the duration of therapy in 'difficult to manage' patients with Genotype 3 HCV would improve response rates and overcome some of the negative factors. Three different research methodologies were used including a meta-analysis of factors associated with treatment failure in patients with Genotype 3 HCV, virological and immunological studies on patients with genotype 3 HCV who had failed to respond to therapy and a clinical trial evaluating extended duration therapy in patients with Genotype 3 HCV infection and cirrhosis.

7.2 Factors influencing the response to therapy in patients with Genotype 3 HCV

The program of research was initiated by a literature review of factors associated with the response to therapy in patients with genotype 3 HCV infection. A number of studies described response rates in patients from Far Eastern Asia (chiefly Taiwan, China and Korea) and these studies suggested that response rates may be at least equal to response rates reported in pivotal clinical studies from America and Europe[5]. However, many such studies combined data from patients with genotype 2 and 3 HCV making it difficult to determine the contribution of each to the overall response rates. Furthermore more recent work has shown that patients from East Asia have a high likelihood of carrying the IL-28 polymorphism that is favourable to virological response in patients with genotype 1 HCV and it is possible that the excellent response rates seen in this population are unduly influenced by this genetic variation. A few studies have been published which prospectively looked at the impact of age >40 years, low pre treatment viral load, cirrhosis and the impact of rapid virological response (RVR) on predicting response to treatment in Pakistani patients [214-216] but the specific effect of ethnicity was not looked at as an independent factor in those studies. One UK study by Freshwater et al suggested that South

Asian ethnicity reduced the response to therapy and this study is the first to clearly document a reduced response in this ethnic group. However the study involved relatively few patients and was not adequately powered to detect confounding variables, such as age, presence of cirrhosis and insulin resistance. Given data from the United States demonstrating that African Americans have a poor response to antiviral therapy we speculated that the findings of Freshwater et al were correct and we attempted to confirm this with a larger study [169].This involved a retrospective review of over 600 patients treated at various Liver Units throughout the UK. This is the first and largest study to examine the impact of South Asian ethnicity on the response to treatment. We collected a cohort of sufficient size and diversity to determine whether or not South Asian ethnicity per se influences the outcome of therapy. This study included all patients who received therapy and thus there was a limited potential for selection bias. However, there were some missing data (which was largely a consequence of the retrospective nature of the study) but there was no evidence of any systematic bias in the data that was not available. Furthermore analysis of the dataset with and without the missing variables did not lead to different conclusions strongly suggesting that the conclusions are robust. Our data show that South Asian ethnicity is not a major factor in reducing response to therapy but cirrhosis, diabetes and older age are important determinants of the treatment response in patients with Genotype 3 HCV. Our analysis of factors influencing the response to anti-viral therapy show that age and the extent of liver fibrosis dominate the outcome and that diabetes is associated with a significant reduction in the SVR rate. Patients were classified as having diabetes if they were registered as diabetic and those without a mention of diabetes were categorised as non-diabetic. There is a possibility that this may have lead to an underestimation of the prevalence of diabetes and formal tests of insulin resistance were not performed.

It is well established that dose reductions and modifications to the treatment regime as a result of treatment related side effects can reduce the efficacy of therapy and it is conceivable that the impact of age and cirrhosis on treatment outcome was related to reductions in the dose of medication. However, the heterogeneous nature of the dose reductions in the different centres over the long time course of this study precluded a full analysis of the impact of dose reduction so we are unable to comment further on the impact of dose changes.

Data from the four UK treatment centres were included in the study. The centres had used different assays to detect viraemia and the threshold varied with time with a cutoff of less than 615 IU/ml being defined as negative in the first few years, which was later reduced to less than 15 IU/ml. For each laboratory, at each time, the local lower limit of detection was accepted, and samples deemed 'negative' by the laboratory were regarded as undetectable. Since studies with Pegylated interferon show that differences in assay sensitivity have minimal impact upon categorisation of outcome we do not believe that these differences were significant in determining the rates of sustained response. However in the determination of RVR it is possible that assay sensitivity did lead to differences in determining whether an RVR had or had not occurred and these data should therefore be treated with some caution.

A number of different treatment regimes were used. In general patients received either 40 kD pegylated interferon α 2a combined with 800 mg of ribavirin or 12 kD pegylated interferon α 2b combined with weight-adjusted ribavirin. Both treatments were normally given for 24 weeks and response guided therapy with a reduction in treatment duration for rapid and early virological responder was not used at the time of this study. In a few centres, some patients were given extended courses of therapy and these were noted. The use of different dosing regimes across different sites may have blurred the affect of ethnicity in predicting the response to treatment in the cohort. Our dataset also included patients from the Freshwater et al study which was part of the Birmingham centre cohort of patients but as the Freshwater et al cohort was also a retrospective review of patients who had received treatment its inclusion in our study is unlikely to have introduced additional bias.Our results indicate that the role of ethnicity in predicting response to treatment was confounded by older age,diabetes and cirrhosis and that S.Asian ethnicity itself did not play a role in determining response to treatment.
In patients with adverse factors that may reduce the response to antiviral therapy clinicians are often tempted to increase the duration of therapy and a number of patients in this cohort did undergo extended therapy. The number of such patients was small and far too few were studied to allow any meaningful comment on the value of extended therapy. The observation that clinicians are using this unproven regime clearly highlights the need for a clinical trial to examine the value of extended duration therapy in patients with Genotype 3 HCV and adverse prognostic factors.

Our study indicates that Genotype 3 HCV is 'easy to cure' provided that the patients are identified when they are young and before they have developed cirrhosis and/or diabetes. These data add further weight to calls for case finding approaches in populations at high risk of Genotype 3 HCV[57].

7.3 Biomarkers of Cirrhosis

Our analysis of factors predisposing to treatment success identified cirrhosis as a strong negative predictor of treatment outcome. We therefore examined diagnostic approaches to the identification of patients with cirrhosis. Many biomarkers have been suggested in the literature with the aim of replacing a liver biopsy. The suggested ideal surrogate marker would be specific for fibrosis of the liver, help in identifying the stage of fibrosis, would not be influenced by co-morbidities (e.g. renal, reticulo-endothelial activation in other organ systems), have a known half-life, a known excretion route and be sensitive and reproducible[179]. The ideal biomarker has not been identified. However meta-analyses of a variety of putative biomarkers has identified panels of serum proteins that may be used to identify fibrosis. Two panels are in common use – the French 'Fibrotest' and the European 'Enhanced Liver Fibrosis test (ELF). Neither of these markers have been evaluated in populations from South Asia and given the high prevalence of Genotype 3 cirrhosis in this population we examined the performance of these markers in this population. The Fibrotest relies upon the serum concentration of bilirubin as a key component of the diagnostic matrix and this element is light sensitive and its concentration degrades with time. We were therefore unable to examine the performance of this marker in our archived samples. We therefore evaluated the performance of the ELF test and of other, non-invasive diagnostic markers in a small cohort of 68 South Asian patients with Genotype 3 chronic HCV infection. These patients were identified retrospectively and we compared the liver biopsy scores with the different surrogate markers i.e. the APRI, ALT/AST ratio, ELF score and FIB-4.It was seen that when using the published cut off values for the ELF test very few patients were correctly identified as being cirrhotic i.e. 5/19 patients. As the initial studies were performed in predominantly Caucasian patients it is possible that the selected cut-off does not apply to this population. If the threshold for the ELF test was adjusted to 9.65 it then correctly identified the cirrhotic patient population (we are grateful to Dr J. Parkes for assistance in calculating this revised cut off value). In our study the correlation between ELF and histology scores remains

significant, but the thresholds for the detection of advanced fibrosis/cirrhosis may need to be revised in individuals of South Asian ethnicity. The other surrogate markers that we evaluated were also validated in Caucasian cohorts and did not perform well in our cohort of patients and once again, may require modification for other patient populations.

There are several problems with our dataset which include the sample size which was very small and comprised of only 68 patients, and there was no confirmatory cohort to validate our modified cutoff value which we propose should be used to diagnose cirrhosis and advanced fibrosis. As most of the patients included in this study had advanced fibrosis/cirrhosis (41% of patients had cirrhosis reflecting the fact that we are a treatment centre specializing in the management of complex patients) further validation of the results should be done in a community based study with a larger proportion of patients with minimal/early disease. It is unclear whether the modified cut off required with the ELF test is a feature of Genotype 3 infection (perhaps caused by its tendency to deposit fat in the hepatocytes) or whether this is a feature of patient race and further studies will be required to address this. Failure of the APRI test to accurately identify cirrhosis in our population group could be due to the use of a 'normal' AST value which is a key component of the APRI test. A cross-sectional study of 411,240 registered blood donors in a Korean population has shown a lower cut off limit for this assay than the conventionally accepted value < 40 U/L but higher than the recently suggested normal ranges of < 30 U/L for males and < 19 U/L for females) [217]. This may indicate that AST values may need to be adjusted in patients from S. Asia. Hence it is unclear which factor (cirrhosis, Genotype 3 , ethnicity or AST value) that leads to the poor performance of the assay. Again larger studies with a confirmatory cohort are needed to validate these results. Similar reasonings apply to the other assays that we investigated and, again, further large scale studies are indicated. Nevertheless despite the shortcomings of our approach this is the first attempt to evaluate commonly used markers of cirrhosis in different ethnic groups and our studies indicate that ethnicity may be an important determinant of the value of a marker of liver fibrosis.

7.4 Duration of therapy in patients with Genotype 3 HCV

Large studies conducted in chronic HCV Genotype 3 patients have recommended that patients should not be considered for shorter treatment duration if they have a high baseline viral load

above 600–800 000 IU /mL, are co infected with HIV or have evidence of advanced fibrosis /cirrhosis[123, 155]. There is evidence that patients with HCV Genotype 3 and higher baseline viral load have lower rates of SVR and higher relapse rates after 24 weeks of treatment than those with lower HCV RNA baseline levels and in patients without RVR, studies have shown that the lowest rates of relapse are obtained with 48 weeks of treatment accompanied by a higher RBV dose [123, 134]. Aghemo et al have shown in their study that patients infected with Genotype 3a who have cirrhosis are 10 times more likely to relapse following treatment with conventional or peginterferon alpha 2a plus RBV than those without cirrhosis[171]. A SVR was less common in cirrhotic (6 of 17) than in non-cirrhotic patients (62 of 74; 35% vs 84%) and by multivariate analysis, cirrhosis was found to be an independent factor for treatment failure in patients chronically infected with HCV 3a and was found to be associated with an increased risk of post-treatment hepatitis relapse[171].A recent study done in Pakistan which included 260 Genotype 3 patients of whom 9 patients were known to have cirrhosis, found that of their population of cirrhotic patients none achieved an SVR with the standard 24 weeks treatment regimen and there was a reduced SVR rate of n=149/260 (57.3%) in these patients compared to the larger studies which have shown a response to treatment of around 75% .There was a relapse rate of 17.69% (n=46/260) in this study. The study was a retrospective review of patients receiving treatment during 2001-2007 where the total patients treated were reviewed and then included /excluded according to the data entered on to the notes. This approach may have led to a source of selection bias. Compliance to treatment was not discussed in the paper.

7.5 Randomised trial of extended duration therapy in patients with G3 HCV and Cirrhosis

To further explore the benefits of extended duration of therapy in patients with Genotype 3 HCV and cirrhosis we conducted a phase IV multicentre, open labelled, randomised study comparing 24 weeks therapy with 40 KD Pegylated interferon alpha 2a and ribavirin (800 mg) to patients randomised to 48 weeks therapy with 40 KD Pegylated interferon alpha 2a and ribavirin (800 mg) in patients with genotype 3 chronic HCV and advanced fibrosis (modified Ishak fibrosis score of 4 or more). This trial was designed so that a total of 140 patients were recruited and randomized (1:1) at baseline to either receive 24 weeks of treatment (Group A) or 48 weeks of therapy (Group B). Both groups received 180µg pegylated interferon alpha 2a in combination with 800mg ribavirin for the treatment duration. A 24 weeks follow-up for both groups was

completed after treatment. This is the first study investigating whether there is any benefit of extending treatment duration in Genotype 3 cirrhotic patients.

The initial exploratory analysis which has been presented in this thesis is restricted to the patients whose data entry is complete (i.e. those who have completed the study and complete data entered on the eCRF). Hence these preliminary findings are subject to change, although the small number of patients currently excluded and the robust nature of the findings make a significant change very unlikely. The study was due to close recruitment in 2010 but due to the slow recruitment process caused by a number of factors which included difficult to identify/ recruit genotype 3 patients with advanced fibrosis/cirrhosis. A total of 16 sites were initially set up out of which two sites were unable to recruit patients. To facilitate recruitment we attempted to enroll patients from an overseas centre - Agha Khan, Karachi, Pakistan was selected and visits to the site were conducted. However contracting difficulties prevented the site being initiated.

The trial was hampered by the change of central laboratories caused by the decision of the original testing laboratory to sell the contract to a second laboratory which then went bankrupt and transferred the work to a third laboratory who proved unable to complete the required study procedures. This change led to local laboratory testing of a number of samples before transfer of the contract to a final approved laboratory who completed the final testing of the study samples. Given the robust nature of the current commercial assays for HCV RNA testing we do not believe that this change led to any significant variation in reporting of HCV RNA values.

According to the initial inclusion/exclusion criteria a number of patient failed screening as they had a platelet count of <70,000 so the protocol was modified and the platelet count was reduced to <30,000 which helped with recruitment of patients and ensured that the study mimicked current prescribing practice. A number of other protocol variations were completed including a change to the time of HBsAg assessment and the withdrawal of the need to convene a safety monitoring committee. We do not believe that these changes could have impacted upon the outcome of the study.

When looking at the virological responses in the study and comparing the response rates in the two treatment arms it was seen that an SVR of 45.31% (n=29/64) in the 24 week treatment arm and a SVR of 43.28% (n= 29/67) in the 48 week treatment arm was achieved. The overall SVR

in the study was 44.27% (n= 58/131). No significant difference between the two treatment arms was found (p = 0.0261) and the trivial difference in response strongly suggests that there is unlikely to be any meaningful difference between the two treatment arms.

Given the data suggesting that extending the duration of therapy may improve response rates in patients who respond slowly to antiviral therapy it was interesting to examine SVR rates in patients who did, or did not, achieve an SVR. Of the 73 patients in the STEPS study who had an RVR, 61% (N-45/73 – 70.6%) achieved an SVR and there was no significant difference between patients with an RVR who achieved an SVR in the group receiving 48 weeks therapy (58.84%) when compared to the group receiving 24 weeks (70.6%). There were fewer patients who did not achieve an RVR and in this group, again there was no significant advantage in SVR in patients receiving 24 or 48 weeks therapy.

We used a fixed dose of Ribavirin of 800mg in the study as this is the licensed dose for this indication. A poor response to treatment was seen in patients with an increased BMI which might be interpreted as indicating that higher doses of ribavirin may be beneficial. To further evaluate whether the poor response was due to using a suboptimal dose of ribavirin in patients who were heavier, we examined whether a weight based ribavirin dosing regime was associated with a better response. We chose the optimal cutoff of Ribavirin dose as >10.66mg/kg as seen in patients with Genotype 1 HCV. We evaluated the patients accordingly with those values. It was seen that patients who were heavier and received a lower ribavirin dose did not achieve the same rates of SVR as the lighter weight patients, but this difference was not found to be statistically significant. Perhaps a future follow on study with a weight based ribavirin dose and its effect on response to treatment in genotype 3 patients with advanced fibrosis/cirrhosis should look at the effect of a higher dose of ribavirin and whether that alters treatment outcome.

There were a total of 29 patients who withdrew from the study - 17 were randomised to the 48 week treatment arm and 12 were assigned to the 24 week treatment arm. In the preliminary interim analysis there was no difference seen in the timing of patients withdrawal in the 48 week treatment arm as 8 dropped out in the initial 24 weeks and 8 dropped out in the remaining 48 weeks of treatment. This suggests that failure to complete the course of therapy because of

adverse events was not responsible for the poor response to extended duration of therapy More patients were withdrawn due to adverse events than withdrew due lack of efficacy and the large number of patients who did not complete therapy confirms previous observations that therapy is poorly tolerated in patients with cirrhosis.

A power calculation was used to define the power of the study and we decided that a benefit of 20% was required as anything less would not be sufficient to justify the additional costs and inconvenience of prolonging therapy. The overall SVR was 44.27% (n-58/131) in the STEPs study. This is in line with our assumptions and our meta-analysis of therapy in patients with Genotype 3 cirrhosis and illustrates the challenges faced in treating this difficult to cure population.

Writing up the trial in retrospect I think that the study was underfunded and the large number of centres with minimal resources for monitoring made the study slow to recruit and led to delays in data entry and delays in data analysis. However despite these practical difficulties the trial has delivered a robust answer to the important question of optimal duration of therapy for patients with Genotype 3 HCV and cirrhosis.

7.6 Virology and immunology of viral relapse in patients with Genotype 3 HCV

We hypothesized that in order to identify and analyse viral and host factors underlying differences between treatment sensitive and treatment refractory groups of patients studies on viral relapse were indicated. Our data, and the data from others, indicates that in patients with Genotype 3 cirrhosis, treatment failure is most often due to relapse following discontinuation of therapy. The mechanisms underlying relapse have not been studied in great detail and the mechanisms underlying relapse remain unclear. To begin to examine mechanisms underlying relapse we collected sequential samples from 30 patients believed to be at high risk of relapse. Such a highly selected cohort clearly carries the risk that the samples may be non-representative but given the effort required to collect sequential samples we chose to use a, potentially, biased sample in the hope of identifying factors associated with relapse which could then be validated in a larger cohort. In twelve patients a virological relapse was observed.

In total 10 patients were followed and T cell population changes observed i.e patients were bled weekly from end of treatment to six weeks post treatment to look for virological relapse and changes in the T regulatory cell population associated with relapse. Early studies done by our group suggested that, in patients with genotype 3 HCV undergoing therapy, when the viral load falls there was an increase in Foxp3 positive T cells [218] perhaps suggesting that these cells may play a hitherto unexpected role in controlling viral replication. We therefore hypothesised that if T regs were involved in suppression of viraemia we should observe changes in T regs following treatment in patients who relapsed and we speculated that these should differ from the changes seen in patients who responded to therapy. Further studies in patients receiving pegylated interferon and ribavirin questioned the original observations and when no significant changes were found in the study population further recruitment of patients for the T cell study was discontinued this line of work.

This was a unique collection of Genotype 3 patients who were followed prospectively in great deal following the cessation of therapy. To study virological changes we studied the 12 patients (of the 30 patients studied) who had relapsed. With the help of a senior scientist Dr. J Waters we were successfully able to PCR amplify early viral samples from five of the patients to look for changes pre and post-treatment. During my limited time in the laboratory I was involved in collecting samples and completing a small number of PCR assays. Further work on my collected samples was later performed by a senior scientist and senior immunologist .The results presented in the chapter is work done by the senior scientists with whom I collaborated. To look for changes in virological sequences pre and post treatment HCV RNA was extracted from the pre-treatment sample and the first HCV-RNA positive post- treatment samples of the relapsed patients and the quasispecies were assessed and compared in 5 patients by sequencing the E2 region, including the HVR1, 2 &3 regions and the PKR-eIF2α region, of 12-15 clones in both pre- and post-treatment samples. I was involved in establishing the PCR based assay but unfortunately I was not successful with the molecular techniques and only limited time was available to address the complex technical issues involved in amplification of highly variable regions of very low titre virus. However subsequent analysis of my samples showed that in 2 patients the quasispecies that emerged post treatment were represented in the pre-treatment sample but in 3 of these patients new quasispecies not present in the pre-treatment samples

emerged suggesting that relapse was due either to the emergence of an interferon resistant virus or to sequestration of virus in a protected site. However due to the small sample size coming to a conclusive result was not possible and this work highlights the difficulties of these studies. Advances in PCR technology and recent developments in deep sequencing may allow this work to be developed further.

7.7 Future Studies

A few studies may arise from the studies in the thesis

- Further studies looking into genotype 3 patient population –looking into increasing treatment duration along with giving patients weight based ribavirin and exploring whether that has a beneficial effect on treatment response.
- Looking into whether IL-28 gene has a role in treatment response in S.Asian genotype 3 patients.
- Evaluating in a larger scale study the effect of quasispecies and whether there is sequence change pre and post treatment in genotype 3 patients in sanctuary sites.
- Whether ELF and other surrogate marker cutoffs need to be verified in S.Asian patients.

Conclusions

This thesis has examined the response to therapy in patients with genotype 3 HCV. We find that the major factor influencing the response to treatment is the extent of liver fibrosis and our data shows that advanced fibrosis alone is responsible for the poor response rates seen in some patient populations. We attempted to identify virological factors associated with treatment failure, specifically relapse, but no such factors were identified in the preliminary studies reported here. To determine approaches to improving the response to therapy in patients with Genotype 3 HCV we completed a randomized controlled clinical trial evaluating two different durations of therapy and we conclude that there is no benefit to extended duration of therapy in this patient population. Thus the mechanisms and optimal management approach for patients with Genotype 3 HCV and cirrhosis remain unclear and it is to be hoped that advances with new direct acting antiviral agents will help to resolve this important clinical problem.

APPENDICES

Appendices 8.1

_St_udy to _E_valuate _P_egasys _S_VR in Genotype 3 HCV infected Cirrhotic Patients

"**STEPS in Geno 3 Cirrhotics**"

Protocol Version 3.6

Dated 21 December 2010

Background and Rationale

Background

Hepatitis C

Hepatitis C virus (HCV) is responsible for a large proportion of chronic liver disease cases worldwide and accounts for 70% of cases of chronic hepatitis in industrialized countries. The global prevalence of chronic hepatitis C (CHC) is estimated to average 3% (ranging from 0.1% to 5%); there are an estimated 170 million chronic carriers worldwide (2.7 million in the USA and 5 million in Western Europe).
HCV demonstrates a high degree of variability and has been classified into six genotypes, with further division into subtypes, based upon the percentage of nucleotide sequence homology. The geographic and epidemiological distributions of the genotypes of HCV are well-documented. Some subtypes, such as 1a, 1b, 2a and 2b, show a worldwide distribution, whereas others, such as 5a and 6a, are relatively restricted to certain geographic regions. At the present time, HCV type 1 is the major genotype and accounts for approximately 40% to 80% of HCV infections overall. In the United States and Europe, both type 1a and 1b are prevalent, with HCV type 2 and type 3 present at a lower frequency.

Study Treatments

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. The recommended regimen for treatment of patients with genotype 3 infection is 180 µg Pegasys given once weekly for 24 weeks in combination with ribavirn 800 mg, given daily.

Pegasys® (Peginterferon alfa-2a, PEG-IFN alfa-2a)

Interferon alfa (IFN) was the first drug shown to have bioactivity against HCV.
Hoffmann-La Roche has chemically modified the interferon alfa-2a molecule by covalently attaching a branched methoxy polyethylene glycol moiety. Pegasys has a decreased systemic clearance rate and an approximately 10-fold increase in serum halflife compared with interferon alfa-2a, and as a result Pegasys circulates in the blood much longer than the parent compound. Subsequent evaluation of Pegasys, 180 µg once weekly (qw), in three large clinical trials in over 1400 patients showed that treatment with Pegasys was more efficacious than treatment with IFN thrice weekly.

Copegus® (Ribavirin)

Copegus (ribavirin) is a guanosine analogue that inhibits the in vitro replication of a wide range of RNA and DNA viruses. The mechanism by which ribavirin acts as an antiviral is not fully defined although it may involve alteration of cellular nucleotide pools and inhibition of viral RNA synthesis. Ribavirin monotherapy has little or no effect on the replication of HCV, but it can result in normalization of serum alanine aminotransferase (ALT) activity and improvement in liver histology. However, relapse occurs in nearly all patients treated with ribavirin alone. Combining Copegus with Pegasys has been found to be more effective than Pegasys monotherapy in the treatment of CHC. In a large clinical trial of 1121 patients, a sustained virological response (SVR) was achieved in 53% of patients treated with Pegasys plus Copegus as compared to 29% of patients treated with Pegasys alone.

Rationale for the Study

Infection with genotype 3 chronic hepatitis C virus (HCV) is common in many parts of the world and is regarded as an 'easy to treat' genotype. Clinical trials have shown that patients with this genotype respond well to therapy and sustained virological response rates in the order of 75% can be achieved. In a randomised controlled clinical trial involving a comparison of 24 and 48 weeks therapy with 40 KD pegylated interferon alfa 2a and 800mg ribavirin patients with genotype 3 HCV responded equally well to 24 and 48 weeks of therapy, indicating that therapy with 24 weeks and a low dose of ribavirin is required to eliminate this virus in a high proportion of those who are infected.

Genotype 3 HCV is common in the Indian sub-continent and, according to recent census figures, over 2 million immigrants from this region are currently living in the UK. Anecdotal reports from a number of different units, including East London and Birmingham indicates that genotype 3 HCV is common in immigrants from Bangladesh and Pakistan and data from East London suggests that a large proportion of these patients have advanced fibrosis. In view of the published studies indicating that 24 weeks therapy is sufficient to eradicate genotype 3 HCV most UK units treat such patients for 24 weeks. However anecdotal and audit data indicates that treatment response rates are reduced in UK patients with genotype 3 chronic HCV and some studies indicate that response rates may be no higher than 50% (GRF, D. Mutimer unpublished observations). There are a number of possible explanations for the poor response to therapy in UK patients with genotype 3 HCV – these include high rates of insulin resistance and obesity and the high prevalence of patients with advanced fibrosis. To begin to address the issues around optimal therapy for patients with genotype 3 HCV we plan a multicentre, randomised clinical trial involving a comparison of 24 and 48 weeks therapy in patients with genotype 3 HCV and advanced fibrosis.

Objectives

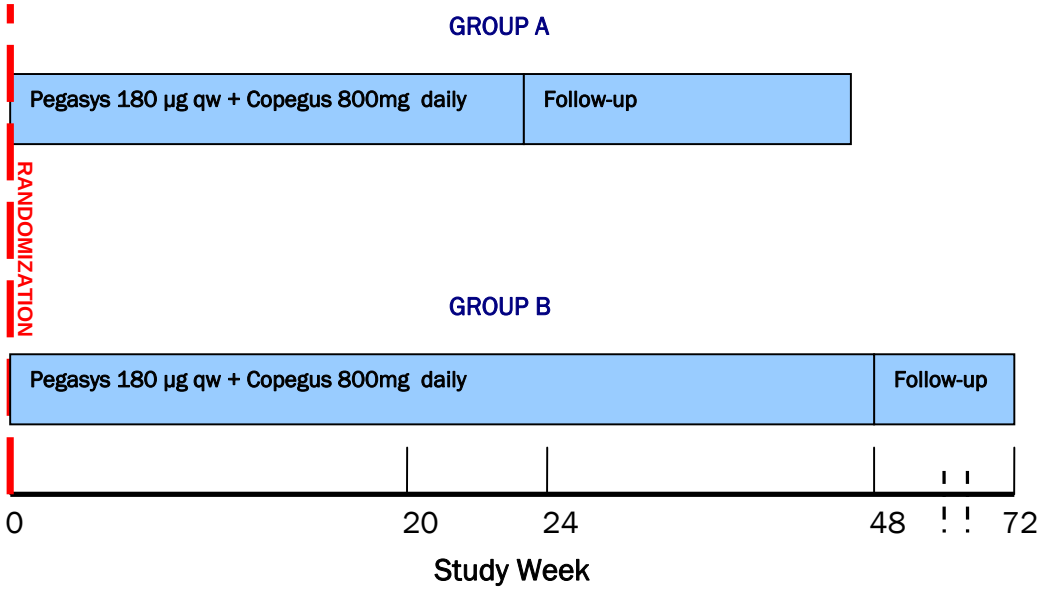
Primary Objectives

- To compare the efficacy and safety of 180µg dosing of Pegasys in combination with Copegus (800mg) for 24 weeks (Group A) with the efficacy and safety of 180µg dosing of Pegasys with Copegus (800mg) for 48 weeks (Group B) in Genotype 3 HCV infected individuals with liver cirrhosis (modified Ishak fibrosis score of equal to or greater than 4 **OR** radiological and/or endoscopic features of cirrhosis)

Secondary Objectives

- To compare the efficacy and safety of Group A compared to Group B based on stratification of HCV RNA viral load at 4 weeks (either <50 copies/ml or ≥50 copies/ml)
- To compare the efficacy and safety of Group A compared to Group B based on stratification of HCV RNA viral load at 12 weeks (either <50 copies/ml or ≥50 copies/ml)
- To evaluate and compare the virological response (HCV RNA) at week 4, 24 (both groups) and week 48 (group B).
- To evaluate (descriptive) baseline characteristics (baseline HCV RNA viral load, insulin resistance, fibrosis score, age and sex) and treatment outcome between Group A and B.

Study Design



* Randomisation to group A or group B is set at baseline.

Number of patients assigned to treatment groups

A total of 140 patients will be randomized (1:1) at baseline to either receive 24 weeks of treatment (Group A) or 48 weeks of therapy (Group B). Both groups will receive 180µg Pegasys in combination with 800mg Copegus for the treatment duration. 24 weeks follow-up for both groups will be completed after treatment.

Centres

The trial will be run as a UK led international trial. The minimum expected randomization per centre is 5. Enrolment will be competitive and recruitment performance will be monitored closely. The sponsor reserves the right to reallocate patients from centres not achieving the expected randomization rate.

Study Drug Management

Study drug (weeks 25 to week 48) will be provided free-of-charge to participating centres, and will be labelled locally for clinical trial use only (study teams at initiation are shown how to order supplies); study drug for weeks 1-24 will come from local/hospital stock and labelled locally: A copy of the label template can be found in the Regulatory Approval section of the pharmacy file. Patients are to be asked to return the used syringes in a locked sharps bin, provided to the patient at dispensing; these will be destroyed according to local SOPs. At the end of the trial, patients will be asked to return all un-used study drug (ribavirin and pegasys) for final reconciliation; the CRF will capture compliance and any discrepancies between returns of un-used study drug and what the patient confirms he/she took. Study teams and pharmacies are to follow sponsor SOPs on IMP management (trial prescription templates, drug accountability/chain of custody, temperature excursion management, drug recall).

Study Population

Under no circumstances are patients who enroll in this study permitted to be re -randomized to this study and enrolled for a second course of treatment.

Overview

Male or females ≥ 18 years old with chronic HCV Genotype 3 infection, with a Liver biopsy within 18 months of study entry showing features of chronic HCV infection and modified Ishak fibrosis score of equal to or greater than 4 **OR** radiological and/or endoscopic features of cirrhosis.

Patients should not have previously been treated with an interferon, ribavirin, viramidine, levovirin, or HCV polymerase or protease inhibitors. Patients with Hepatitis B or HIV co-infection are excluded. Also patients with other forms of liver disease, anaemia, hepatocellular carcinoma, pre-existing severe depression or other psychiatric disease, cardiac disease, renal disease, uncontrolled seizure disorders, or severe retinopathy are excluded based on protocol criteria.

Inclusion Criteria

- Age ≥18 years of age
- Chronic genotype 3 HCV infection as evidenced by HCV antibody and RNA positivity with genotype 3 infection confirmed at a central laboratory.
- Liver biopsy within 18 months of entry showing features of chronic HCV infection and modified Ishak fibrosis score of equal to or greater than 4 OR radiological and/or endoscopic features of cirrhosis.
- HBsAg negative result within the past 2 years (2 years from screening),
- No clinical evidence of co-infection with HIV
- Platelet count ≥ 30,000 cells/mm^3, Neutrophil count > 600 cells/mm^3
- Compensated liver disease (Child-Pugh Grade A clinical classification – Appendix 2)
- Negative urine pregnancy test result (for females of childbearing potential) documented within the 24-hour period prior to the first dose of study drugs. Additionally, all female patients of childbearing potential and all males with female partners of childbearing potential must 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

Exclusion Criteria

- Previous therapy for chronic HCV infection -interferon alpha (IFN), PEG-IFN, ribavirin, viramidine, levovirin, or investigational HCV protease or polymerase inhibitors
- Patients who are expected to need alternative antiviral therapy with established or perceived activity against HCV at any time during their participation in the study are also excluded
- Evidence of other cause of significant liver disease – serum ferritin > 1000, biochemical evidence of Wilson's disease, autoantibody titres in excess of 1:160
- Platelet count <30,000 cells/mm^3, Neutrophil count ≤ 600 cells/mm^3
- Poorly controlled diabetes that, in the opinion of the investigator, precludes therapy
- Severe retinopathy that, in the opinion of the investigator, precludes therapy
- Decompensated cirrhosis (Childs Pugh B or C – Appendix 2)
- The use of colony stimulating factors such as granulocyte colony stimulating factor G-CSF), erythropoietin or other therapeutic agents to elevate haematology parameters to facilitate patient entry into the study

- Haemoglobin concentration <10 g/dL in females or <10 g/dL in males or any patient with a baseline increased risk for anaemia (eg, thalassemia, sickle cell anaemia, spherocytosis, history of gastrointestinal bleeding) or for whom anaemia would be medically problematic

- Females who are pregnant or breast-feeding

- History of severe psychiatric disease that, in the opinion of the investigator, precludes therapy with pegylated interferon

- History of immunologically mediated disease (eg, inflammatory bowel disease, idiopathic thrombocytopenic purpura, lupus erythematosus, autoimmune haemolytic anemia, 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

- History of severe cardiac disease (eg, NYHA Functional Class III or IV, myocardial infarction within 6 months, ventricular tachyarrhythmias requiring ongoing treatment, unstable angina or other significant cardiovascular diseases). In addition, patients with documented or presumed coronary artery disease or cerebrovascular disease should not be enrolled if, in the judgment of the investigator, an acute decrease in haemoglobin by up to 4 g/dL (as may be seen with ribavirin therapy) would not be well-tolerated

- History of uncontrolled severe seizure disorder

- Evidence of an active or suspected cancer or a history of malignancy within the last 2 years. Patients with a lesion suspicious for hepatic malignancy on an imaging study will be eligible only if the likelihood of carcinoma is ≤10% following an appropriate evaluation

- History of any systemic antineoplastic or immunomodulatory treatment (including supraphysiologic doses of steroids or radiation) ≤6 months prior to the first dose of study drug or the expectation that such treatment will be needed at any time during the study

- Other on-going serious medical condition in the opinion of the investigator that would prohibit treatment with Pegasys or Copegus

- Poorly controlled thyroid dysfunction

- History of major organ transplantation with an existing functional graft

- Unable or willing to provide informed consent

- History of having received investigational drug 3 months prior to 1st dose of study drug

Concomitant Medication and Treatment

Systemic antiviral treatments with established or perceived activity against HCV, antineoplastic and immunomodulatory treatments (including steroids at supraphysiologic doses and radiation) are not allowed during the study. Steroids given at physiologic doses (defined as topical/inhaled

steroids for an indefinite duration, steroid replacement therapy at doses calculated to mimic normal serum levels for an indefinite duration, or oral short course pulse therapy equivalent to using up to 30 mg of prednisone daily for 7 days) are permitted. Other remedies being taken by the patient for possible or perceived effects against HCV, such as herbal agents, are excluded, as are other investigational drugs.

The total daily dose of acetaminophen (paracetamol) should not exceed 4 grams per day. A complete listing of all concomitant drugs received must be recorded in the CRF.

Concomitant medications which are evident for an AE that is stated (expected) in the SmPC will not be captured in the eCRF, however if this AE becomes an SAE they will be reported to the sponsor (as per page 21 of this protocol). For AEs that are listed in the current SmPC, yet the investigator feels the sponsor should be aware of these, then the investigator should record these in the eCRF.

Under no circumstances are patients to be treated with growth factors during screening to elevate haematology laboratory parameters to facilitate entry into the study. However, use of growth factors, eg, erythropoietin and G-CSF, will be allowed during the study if considered medically necessary by the investigator.

Alcohol consumption is to be strongly discouraged. During the study, patients should consume no more than an average of 20 g of alcohol daily. This is because of possible adverse effects of alcohol on the response to therapy with IFNs in patients with CHC.

Dose reductions

Therapy with pegylated interferon and ribavirin may lead to anaemia and reductions in platelet and neutrophil count. These are commonly seen during therapy and do not normally require any dose modification. However severe or clinically significant reductions in haemoglobin or platelet/neutrophil counts may require a reduction in either the dose of ribavirin or the dose of pegylated interferon. Such dose adjustments are permissible during the study and should be performed at the discretion of the treating clinician. All dose adjustments should be recorded in the eCRF.

Criteria for Premature Withdrawal

Patients have the right to withdraw from the study at any time for any reason. The investigator also has the right to withdraw patients from the study in the event of intercurrent illness, adverse events, insufficient therapeutic response, protocol violations, administrative reasons or other reasons. An excessive rate of withdrawals can render the study interruptible; therefore, unnecessary withdrawal of patients should be avoided. Should a patient decide to withdraw, all efforts should be made to complete and report relevant observations as thoroughly as possible.

The investigator should contact the patient or a responsible relative either by telephone or through a personal visit to determine as completely as possible the reason for the withdrawal. If the reason for withdrawal of a patient from the study is an adverse event or an abnormal laboratory test result, the specific event or test should be recorded on the CRF.

All patients who discontinue from treatment prematurely should be assessed for safety at 4 weeks and again at 12 weeks post their last dose of study medication.

Patients who discontinue from treatment prematurely and whose HCV RNA is undetectable at last HCV RNA assessment (4 weeks post last dose or other) are expected to have follow-up assessments at 12 weeks and 24 weeks post their last dose of study medication. 24 week post dose viral load is to be sent to the central laboratory.

Screening Examination and Eligibility screening Form

All patients must provide written informed consent before any study specific assessments or procedures are performed. An eligibility screening form (ESF) documenting the assessment of each screened patient with respect to each of the protocol's inclusion and exclusion criteria is to be completed by the investigator. The completed ESFs should be retained with the study documents at the site. Patients who fulfil all of the inclusion and none of the exclusion criteria will be accepted into the study.

The following screening assessments must be obtained within 35 days prior to the initiation of test drug administration.

Procedure of Enrolment of Eligible Patients

Only patients who fulfil all entry criteria are eligible for enrolment into the study. Each patient will be designated a trial number at baseline. The trial number is obtained by phoning the sponsor after successful screening is completed.

The patient randomisation numbers will be generated by the sponsor or its designee. A patient's treatment assignment will be given to the investigator over the telephone at the time of individual patient randomisation.

The randomisation of each patient to either 24 weeks of treatment or 48 weeks of treatment will be set at baseline.

The electronic master randomisation list will be kept in a central repository maintained by the sponsor. Randomisation of patients according to the master randomisation list will be administered by the trial manager. Randomisation codes will not be available at the study centre or to the project statisticians. The randomisation list will be kept securely and have a full audit trail of changes.

Clinical and Laboratory Assessments of Procedures

Central Laboratory Manual: *Study teams/sites are to adhere to the sponsor's SOP for sample collection, processing, storage (pre-transit) and shipping.*

Local Laboratory Tests: Study teams are to adhere to sponsors SOP on sample chain of custody for both local and central laboratory samples and results. Local sites are to ensure the maintenance of lab compliance to GCP and to provide the sponsor with relevant documentation.

Refer to appendix 5 for sample timepoints and requirements (and protocol section 'Criteria for Premature Withdrawal' – for any early withdrawals).

Efficacy Assessments

Serum HCV RNA will be assessed by PCR techniques and monitored for efficacy.
Quantitative HCV RNA titres will be obtained throughout the treatment period and the 24-week follow-up period (post treatment) according to the Schedule of Assessments.

Patients who discontinue from treatment prematurely and whose HCV RNA is undetectable at last HCV RNA assessment (4 weeks post last dose or other) are expected to return for HCV RNA assessments at 24 weeks post their last dose of study medication.

This study will employ a serum bank in the event that some tests need to be repeated.

Safety Assessments

Safety assessments will be performed throughout the treatment period and the 24-week follow-up period (post treatment) according to the Schedule of Assessments.

Measurements of safety include:

- Vital signs consisting of systolic and diastolic blood pressure, and pulse rate
- Local safety bloods as per appendix
- Clinical adverse events
- Depression assessments as per local standard care/practice

Assessments – timing of visits

Patients should be assessed as indicated in the Schedule of Visits. In the event that a patient is unable to attend on a particular day the timing of the visit may be changed so that the visit is completed either 3 days before or 3 days after the scheduled visit.

Please refer to appendix 5 for the Schedule of Assessments/Visits!

Safety Instructions and Guidance

Adverse Events (AEs) and laboratory abnormalities

Clinical AEs

Per the International Conference on Harmonisation (ICH), an AE is any untoward medical occurrence in a patient or clinical investigation subject administered a pharmaceutical product and which does not necessarily have a causal relationship with this treatment. An AE can therefore be any unfavourable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated

with the use of a medicinal (investigational) product, whether or not considered related to the medicinal (investigational) product. Pre-existing conditions which worsen during a study are to be reported as AEs.

All clinical adverse events encountered, which are not noted in the current SmPC, during the clinical study will be reported on the AE form of the CRF. Concomitant medications which are for an AE that is stated in the SmPC will not be captured in the CRF.

Intensity

Intensity of AEs will be graded by the investigator using the four-point scale (mild, moderate, severe, life-threatening) below and reported in detail on the CRF.

Mild	discomfort noticed but no disruption of normal daily activity
Moderate	discomfort sufficient to reduce or affect daily activity
Severe	inability to work or perform normal daily activity
Life Threatening	represents an immediate threat to life

Drug Adverse Event Relationship

Relationship of the AE to the treatment should always be assessed by the investigator.
Description of scales can be found in Appendix 1.

Serious Adverse Events [Immediately Reportable to the Sponsor]

A serious adverse event is any experience that suggests a significant hazard, contraindication, side effect or precaution. It is any AE that at any dose fulfils at least one of the following criteria:

- is fatal; [results in death; NOTE: death is an outcome, not an event]
- is life-threatening (NOTE: the term "life-threatening" refers to an event in which the subject was at immediate risk of death at the time of the event; it does not refer to an event which could hypothetically have caused a death had it been more severe).
- required in-patient hospitalization or prolongation of existing hospitalization;
- results in persistent or significant disability/incapacity;
- is a congenital anomaly/birth defect;
- is medically significant or requires intervention to prevent one or other of the outcomes listed above.

Any clinical adverse event or abnormal laboratory test value that is serious occurring during the course of the study, irrespective of the treatment received by the patient, must be reported to the sponsor within 1 working day of occurrence (expedited reporting).

The full requirements of the ICH Guideline for Clinical Safety Data Management, Definitions and Standards for Expedited Reporting, Topic E2 will be adhered to (see Appendix 4).

SAEs/SUSARs reporting will be followed, by the sponsor, in accordance with the European Directive 2001/20/EC

Treatment and follow-up of AEs

AEs, especially those for which the relationship to test drug is “related,” should be followed up until they have returned to baseline status or stabilized. If a clear explanation is established it should be recorded on the CRF. If the use of medications excluded by the protocol is deemed necessary, the patient may need to be discontinued from the test regimen after consultation with the sponsor.

Laboratory Test Abnormalities

Laboratory test results will be recorded on the laboratory results form of the CRF, or appear on electronically produced laboratory reports submitted directly from the central laboratory, if applicable.

Laboratory test value abnormalities should not be reported as adverse events on the "Adverse Event" form of the CRF unless the abnormal laboratory assessment meets any one of the criteria listed below:

- is considered to be an SAE
- results in discontinuation from study treatment
- results in a requirement for a change in concomitant therapy (eg, addition of, interruption of, discontinuation of, or any other change in a concomitant medication, therapy or treatment)

Follow-up of Abnormal Laboratory Test Values

In the event of medically significant unexplained abnormal laboratory test values, the tests should be repeated and followed up until they have returned to the normal range and/or an adequate explanation of the abnormality is found. If a clear explanation is established it should be recorded on the CRF.

Handling of Safety Parameters

Reporting of AEs

All clinical adverse events encountered, which are not noted in the current SmPC, during the clinical study will be reported on the AE form of the CRF. Concomitant medications which are for an AE that is stated in the SmPC will not be captured in the CRF.

Information regarding the intensity of the AE, its relationship to study treatment, and any interventions required to treat the AE must be provided. The duration of an AE is the time elapsed from when the patient begins to experience symptoms (or the AE is identified by the patient or investigator) to when the AE is considered to be resolved.
Any AE that occurs, then stops, and then reoccurs within a 28-day window must be recorded as “intermittent” on the AE form of the CRF.

Reporting of Serious Adverse Events [immediately reportable]

Any clinical AE or abnormal laboratory test value which is *serious* and which occurs during the course of the study (as defined above), regardless of the treatment group, must be reported to the sponsor **within 24 hours** of the investigator becoming aware of the event (expedited reporting).

All SAEs, regardless of relationship to test medication, must be reported through the 24 week period after stopping the last dose of trial medication. It is not considered necessary to report SAEs or deaths that occur greater than 24 weeks after the last dose of test medication, unless the SAE or death is considered related to test medication.

Related serious adverse events ***MUST*** be collected and reported regardless of the time elapsed from the last study drug administration, even if the study has been closed.

The definition and reporting requirements of **ICH Guideline for Clinical Safety Data Management, Definitions and Standards for Expedited Reporting, Topic E2** will be adhered to. Complete information can be found in Appendix 3.

Pregnancy

A female patient must be instructed to stop taking study medications and immediately inform the investigator if she becomes pregnant during the study. Pregnancies occurring up to 6 months after the completion of the test drug must also be reported to the investigator. The investigator should report all pregnancies within 24 hours to the sponsor. The investigator should counsel the patient and discuss the risks of continuing with the pregnancy and the possible effects on the foetus. Monitoring of the patient should continue until conclusion of the pregnancy.

Pregnancy occurring in the partner of a male patient participating in the study should also be reported to the investigator and the sponsor immediately. The partner should be counselled and followed as above. Such patients may not continue to receive Copegus.

Warnings and Precautions

PEG-IFN alfa-2a

Alpha interferons, including Pegasys, may cause or aggravate fatal or life-threatening neuropsychiatric, autoimmune, ischemic, and infectious disorders. Patients should be monitored closely with periodic clinical and laboratory evaluations. Therapy should be withdrawn from patients with persistently severe or worsening signs or symptoms of these conditions. In many, but not all cases, these disorders resolve after stopping Pegasys therapy.To date, no teratology or reproduction studies have been conducted in humans with Pegasys. Primate teratology studies indicate an increased incidence of spontaneously aborted foetuses in pregnant rhesus monkeys (*Macaca mulatta*) receiving high doses of intramuscular Roferon®-A. Studies with Roferon®-A in nonpregnant rhesus monkeys have shown menstrual cycle irregularities, including prolonged menstrual periods. Male fertility and teratological evaluations have yielded no significant adverse effects to date. Interferons are human proteins which show a substantial

degree of species specificity, making extrapolation of animal study data to humans of questionable value. Investigations have been conducted in normally cycling healthy females using nonrecombinant human leukocyte interferon. Results demonstrate a significant reduction of serum estradiol and progesterone concentrations during the treatment interval. There are no adequate, controlled studies of any IFN in pregnant females.

Ribavirin

Ribavirin is a known teratogen (see Pregnancy Category X). Therefore, extreme care must be taken to avoid pregnancy during the study in female patients, and female partners of male patients. **RIBAVIRIN THERAPY SHOULD NOT BE INITIATED UNLESS A REPORT OF A NEGATIVE PREGNANCY TEST HAS BEEN OBTAINED IMMEDIATELY PRIOR TO INITIATION OF THERAPY**. Fertile patients will be excluded from this study unless they are using two reliable forms of contraception during the treatment period and follow-up. Females who are pregnant or lactating will also be excluded. A pregnancy test will be performed on each female of childbearing potential prior to entry into the study and must be performed by the patient every 4 weeks thereafter during the study. Moreover, fertile females should continue to use effective birth control and perform a urine pregnancy test on a monthly basis for 6 months following their last dose of ribavirin.

Pregnancy Category X

Ribavirin has significant teratogenic and/or embryocidal potential in all test animals.
Malformations of the skull, palate, eye, jaw, limbs, skeleton, and GI tract were observed, the incidence and severity of teratogenic effects increasing with escalation of the drug dose. Survival of foetuses and offspring was reduced. In conventional embryotoxicity and teratogenicity studies in rats and rabbits, no effect dose levels were well below those for proposed clinical use. Therefore, in order to avoid pregnancy, females of childbearing age and males should use two reliable forms of effective contraception (combined) throughout the entire period of the study (treatment and follow-up): these may include, but are not limited to, birth control pills, intrauterine devices, condoms, diaphragms, implants, surgical sterilization, or being in a post-menopausal state. Because ribavirin may be transmitted in semen, it is recommended that one method of contraception be a barrier-type, e.g., condom. If pregnancy occurs in a patient or partner of a patient during the treatment period, the cases should be handled as described in (“Pregnancy”).

The Patient Informed Consent form will include this information with special attention to the potential risk to the foetus should conception occur while the patient or partner is participating in ribavirin trials.

Carcinogenesis and Mutagenesis

Although the literature and sponsor-conducted studies indicate that ribavirin is genotoxic, results from the 6-month carcinogenicity study in p53 (+/-) knockout mice revealed no evidence of treatment-related neoplasia. Results from a study in rats indicated that ribavirin was not oncogenic at the maximum tolerated dose of 60 mg/kg daily.

Adverse Reactions

The primary toxicity of ribavirin is anaemia. Reduction in haemoglobin levels generally occurs within the first 1 to 2 weeks of initiating therapy. Cardiac and pulmonary events associated with anaemia may occur. Ribavirin should be administered with caution to patients with pre-existing cardiac disease. Patients should be assessed before commencement of therapy and should be appropriately monitored during therapy. If there is any deterioration of cardiovascular status, therapy should be stopped. In addition, ribavirin is contraindicated in patients with a history of hypersensitivity to ribavirin.

Statistical Consideration and analytical plan

Primary and secondary study endpoints

Primary efficacy endpoints

The primary measure of efficacy will be SVR defined as the percentage of patients with undetectable HC RNA (<50 IU/ml) in group A (24 weeks of treatment) compared to Group B (48 weeks of treatment).

All HCV RNA viral load measurements will be conducted with the Roche TaqMan HC test.

Secondary efficacy endpoints

- SVR in group A and B Stratified by HCV viral load after 4 weeks of therapy (either <50 IU/ml or ≥ 50 IU/ml)
- SVR in group A and B Stratified by HCV viral load after 12 weeks of therapy (either <50 IU/ml or ≥ 50 IU/ml)
- Virological response at 4 weeks in Group A & B
- Virological response at 12 weeks in Group A & B
- Virological response at 24 weeks in Group A & B
- Virological response at week 48 in Group B
- Virological response in Group A + B by baseline parameters (Age, baseline fibrosis, baseline viral load)

All HCV RNA viral load measurements will be conducted with the Roche TaqMan HC test.

Safety Endpoints

The following safety parameters will be evaluated: adverse events, laboratory test results and vital signs.

Statistical and Analytical Methods

Statistical model and Sample Size

Audit data from participating centres suggests that sustained virological response rates of approximately 50% may be expected in this population with difficult to treat genotype 3 chronic HCV. To justify the additional expense and side effects of therapy an increase in sustained virological response rates to 75% will be required. Based on standard statistical criteria a total of 60 patients per treatment arm will be required to provide a >80% chance of detecting a significant difference. Assuming that 10% of patients will withdraw from the study a total of 66 patients per treatment arm will be required and we will therefore aim to recruit 70 patients per arm.

Analysis Populations

The four populations to be analyzed in this study are:

All patients randomized population includes all patients randomized. Patients will be analyzed according to the treatment group to which they were assigned at randomization regardless of the treatment they actually received after randomization.
All patients treated population includes all patients randomized who received at least one dose of either study medication after screening. This population will also be referred to as the **intent-to-treat (ITT) population**. (ITT)
Per-protocol population excludes a randomized patient if the patient meets any of the exclusion criteria. Analyses using the per-protocol population will be performed only if >10% of patients are excluded from the ITT population.
Safety population includes only patients who receive at least one dose of either study medication and have at least one post baseline safety assessment. Patients will be analyzed according to the treatment they actually received, regardless of whether they discontinue prior to week 20.

Efficacy Analysis

ITT Analysis

All primary and secondary efficacy endpoints will be analyzed using the ITT population.

Per-Protocol Analysis

All primary and secondary efficacy endpoints will also be analyzed using the per protocol population if >10% of patients are excluded from the ITT population.

Criteria for Exclusion of Data from Analysis

Patients who are successfully screened but never receive any study medications will be excluded from the ITT analyses.

Patients who are randomized will be considered major protocol violators and will therefore be excluded from the per-protocol analyses if they meet any of the criteria listed below:

1. Never received any study medications.
2. Infection with any single HCV genotype other than genotype 3, infection with HIV
3. History of having received alpha IFNs, PEG-IFN, ribavirin, viramidine, levovirin, or investigational HCV protease or polymerase inhibitors at any previous time, or any other systemic antiviral therapy with established or perceived activity against the hepatitis C virus ≤3 months prior to the first dose of study drug.
4. History of having received any investigational drug 3 months prior to the first dose of study drug.
5. Patients with no HCV RNA assessment pre-baseline.

Safety Analysis

All patients who have received at least one dose of study medication and have had at least one post baseline safety assessment will be included in the safety analysis. In the analyses of safety data, all adverse events or abnormal laboratory findings occurring during treatment and the untreated follow-up period will be summarized.

AEs will be assigned preferred terms and categorized into body systems according to the Medical Dictionary for Regulatory Affairs (MedDRA). The proportion of patients who experienced AEs will be calculated by dividing the number of patients who experienced the AE during the treatment or follow-up period by the number of evaluable patients in the safety analysis. AEs will be summarized by treatment group and by body system and by event within each body system.

Laboratory data will be analyzed according to UK standards. All lab data will be converted to the Système International d'Unités (SI) units for reporting and processing purposes. For those lab tests lacking sufficiently common procedures, in order to use a universal "normal range," transformations of scale will be executed at the time of conversion to SI units, using the investigator or central lab supplied reference range in transforming to a common accepted range. This will be done for a limited set of tests (that is, the enzyme tests), and in almost all instances the investigator lower limit will be supplanted by zero.

Individual patient values of vital sign parameters including systolic and diastolic blood pressure and heart rate will be listed by patient. The absolute and percentage changes from baseline will be computed and potentially serious changes will be flagged. Appropriate summary statistics will be provided for all vital sign parameters. For a change from baseline in any vital sign parameter to be considered potentially serious, the change must be an increase or decrease of greater than 20% in the direction of worsening and the absolute value must be outside the corresponding reference range as shown below:

• Systolic Blood Pressure <85 or >180 mmHg OR Diastolic Blood Pressure >110 mmHg

• Heart Rate <50 or >120 beats per minute

Exploratory Analysis

Exploratory analyses using logistic regression models will be performed to examine the effect of demographic and baseline disease characteristics on the probability of SVR.

Safety Review Board

Safety data will be summarized on a regular basis and reviewed by an SRB. Safety data to be generated will include summaries and listings of adverse events, dose modifications, premature discontinuations, adverse events or laboratory abnormalities leading to premature discontinuation, and marked laboratory abnormalities.

ETHICS AND GENERAL STUDY ADMINISTRATION

ETHICAL ASPECTS

Local Regulations/Declaration of Helsinki

The investigator will ensure that this study is conducted in full conformance with the principles of the "Declaration of Helsinki" or with the laws and regulations of the country in which the research is conducted, whichever affords the greater protection to the individual. The study must fully adhere to the principles outlined in "Guideline for Good Clinical Practice" ICH Tripartite Guideline [January 1997] or with local law if it affords greater protection to the subject. For studies conducted in the EU/EEA countries, the investigator will ensure compliance with the EU Clinical Trial Directive [2001/20/EC].

Informed Consent

It is the responsibility of the investigator, or a person designated by the investigator [if acceptable by local regulations], to obtain written informed consent from each subject participating in this study, after adequate explanation of the aims, methods, anticipated benefits, and potential hazards of the study. For subjects not qualified or incapable of giving legal consent, written consent must be obtained from the legally acceptable representative. In the case where both the subject and his/her legally acceptable representative are unable to read, an impartial witness should be present during the entire informed consent discussion. After the subject and representative have orally consented to participation in the trial, the witness' signature on the form will attest that the information in the consent form was accurately explained and understood. The investigator or designee must also explain that the subjects are completely free to refuse to enter the study or to withdraw from it at any time, for any reason. The eCRFs for this study contain a question for documenting informed subject consent, and this must be completed appropriately. If new safety information results in significant changes in the risk/benefit assessment, the consent form should be reviewed and updated if necessary. All subjects [including those already being treated] should be informed of the new information, given a copy of the revised form and give their consent to continue in the study.

Independent Ethics Committees/Institutional Review Board

***Independent Ethics Committees*:** This protocol and any accompanying material provided to the subject [such as subject information sheets or descriptions of the study used to obtain informed consent] as well as any advertising or compensation given to the patient, will be submitted by the investigator to an Independent Ethics Committee. Approval from the committee must be obtained before starting the study, and should be documented in a letter to the investigator specifying the date on which the committee met and granted the approval. Any modifications made to the protocol after receipt of the Independent Ethics Committee approval must also be submitted by the investigator to the Committee in accordance with local procedures and regulatory requirements. When no local review board exists, the investigator is expected to submit the protocol to a regional committee. If no regional committee exists, Roche will assist the investigator in submitting the protocol to the European Ethics Review Committee.

CONDITIONS FOR MODIFYING THE PROTOCOL

Protocol modifications to ongoing studies must be made only after consultation between an appropriate representative of the sponsor and the investigator [investigator representative[s] in the case of a multicenter trial]. Protocol modifications must be prepared by a representative of the sponsor. All protocol modifications must be submitted to the appropriate Independent Ethics Committee or Institutional Review Board for information and approval in accordance with local requirements, and to Regulatory Agencies if required. Approval must be awaited before any changes can be implemented, except for changes necessary to eliminate an immediate hazard to trial subjects, or when the change[s] involves only logistical or administrative aspects of the trial [e.g. change in monitor[s], change of telephone number[s].

CONDITIONS FOR TERMINATING THE STUDY

Both the sponsor and the investigator reserve the right to terminate the study at any time. Should this be necessary, both parties will arrange the procedures on an individual study basis after review and consultation. In terminating the study, the sponsor and the investigator will assure that adequate consideration is given to the protection of the patient’s interests.

STUDY DOCUMENTATION, CRFS AND RECORD KEEPING

Investigator's Files / Retention of Documents

The Investigator must maintain adequate and accurate records to enable the conduct of the study to be fully documented and the study data to be subsequently verified. These documents should be classified into two different separate categories **(1) Investigator's Study File, and (2) subject clinical source documents**. The Investigator's Study File will contain the protocol/amendments, Independent Ethics Committee/Institutional Review Board and governmental approval with correspondence, sample informed consent, drug records, staff curriculum vitae and authorization forms and other appropriate documents/correspondence etc. In addition at the end of the study the investigator will receive the patient

data, which includes an audit trail containing a complete record of all changes to data, query resolution correspondence and reasons for changes, in human readable format on CD which also has to be kept with the Investigator's Study File.

Subject clinical source documents [usually defined by the project in advance to record key efficacy/safety parameters independent of the CRFs] would include patient hospital/clinic records, physician's and nurse's notes, appointment book, original laboratory reports, electrocardiogram, electroencephalogram, X-ray, pathology and special assessment reports, signed informed consent forms, consultant letters, and subject screening and enrolment logs. The Investigator must keep these two categories of documents (including the archival CD) on file for at least 15 years after completion or discontinuation of the study. After that period of time the documents may be destroyed, subject to local regulations.

Should the Investigator wish to assign the study records to another party or move them to another location, the sponsor must be notified in advance. If the Investigator can not guarantee this archiving requirement at the investigational site for any or all of the documents, special arrangements must be made between the Investigator and the sponsor to store these in a sealed container[s] outside of the site so that they can be returned sealed to the Investigator in case of a regulatory audit. Where source documents are required for the continued care of the patient, appropriate copies should be made for storing outside of the site.

Source Documents and Background Data

The investigator shall supply the sponsor on request with any required background data from the study documentation or clinic records. This is particularly important when errors in data transcription are suspected. In case of special problems and/or governmental queries or requests for audit inspections, it is also necessary to have access to the complete study records, provided that patient confidentiality is protected.

Audits and Inspections

The investigator should understand that source documents for this trial should be made available to appropriately qualified personnel from the sponsor, or to health authority inspectors after appropriate notification. The verification of the CRF data must be by direct inspection of source documents.

Case Report Forms (eCRF)

Data for this study will be captured via electronic CRFs . The investigator will for each patient randomised manage the completion of eCRF data entry -investigator or authorized delegated study staff to complete data entry. This also applies to records for those patients who fail to complete the study (even during a pre-randomization screening period if a CRF was initiated). If a patient withdraws from the study, the reason must be noted on the eCRF. If a patient is withdrawn from the study because of a treatment-limiting adverse event, thorough efforts should be made to clearly document the outcome. The investigator should ensure the accuracy, completeness, and timeliness of the data reported to the sponsor in the eCRFs and in all required reports.

MONITORING THE STUDY

It is understood that the responsible sponsor designee will contact and visit the investigator as per the sponsor's monitoring SOP, on request, to inspect the various records of the trial (eCRF data and other pertinent data) provided that patient confidentiality is maintained in accord with local requirements. It will be the sponsor's project teams responsibility to inspect the eCRF database at regular intervals throughout the study, to verify the adherence to the protocol and the completeness, consistency and accuracy of the data being entered on them. The monitor should have access to laboratory test reports and other patient records needed to verify the entries on the eCRF. The investigator (or his/her deputy) agrees to cooperate with the monitor to ensure that any problems detected in the course of these monitoring visits are resolved.

CONFIDENTIALITY OF TRIAL DOCUMENTS AND SUBJECT

RECORDS

The investigator must assure that subjects' anonymity will be maintained and that their identities are protected from unauthorized parties. On eCRFs or other documents submitted to the sponsor, subjects should not be identified by their names and to adhere to the sponsor's/trial confidentiality SOP (see appendix 7). The investigator should keep a subject enrolment log showing codes and names. The investigator should maintain documents not for submission to the sponsor, e.g., subjects' written consent forms, in strict confidence. Study sites and subcontractors are to adhere to sponsor SOPs on confidentiality.

PUBLICATION OF DATA AND PROTECTION OF TRADE SECRETS

The results of this study may be published or presented at scientific meetings. If this is foreseen, the investigator agrees to submit all manuscripts or abstracts to the sponsor prior to submission. In accord with standard editorial and ethical practice, the sponsor will generally support publication of multicentre trials only in their entirety and not as individual centre data. Authorship will be determined by mutual agreement.

Appendix 1

AEs Categories for Determining Relationship to Test Drug

Un-Related:

Evidence exists that the adverse event has an etiology other than the study drug. For example a pre-existing condition, underlying disease, intercurrent illness or concomitant medication.

Related:

A temporal relationship exists between the adverse event (AE) onset and administration of the study drug that cannot be readily explained by the subject's clinical state or concomitant therapies. Furthermore, the AE appears with some degree of certainty to be related, based on the known therapeutic and pharmacologic actions or adverse event profile of the study drug. In case of cessation or reduction of the dose, the AE abates or resolves and reappears upon rechallenge.

Appendix 2

Child-Pugh classification of severity of liver disease

	Points Scored for Increasing Abnormality		
Clinical and Biochemical Measurements	**1**	**2**	**3**
Encephalopathy (grade)[a]	None	1 or 2	3 or 4
Ascites[b]	Absent	Slight	Moderate
Bilirubin (mg/100 mL)	<2	2 - 3	>3
Albumin (g /100 mL)	>3.5	2.8 - 3.5	<2.8
Prothrombin time (International Nomalized Ratio[c])	<1.7	1.7- 2.3	>2.3

a) According to grading of Trey, Burns and Saunders (1966)
b) As determined by physical examination alone
c) Prothrombin time results should be reported and used for calculations only as International Normalized Ratios (INR), because of variations in the methods used and reference ranges for controls (expressed in seconds).

1, 2 or 3 points are scored for increasing abnormality of each of the 5 parameters measured.

Grade A: 5 or 6

Grade B: 7 to 9

Grade C: 10 to 15

Appendix 3

ICH Guidelines for Clinical Safety Data management

Definitions and Standards for Expedited Reporting

A serious adverse event is any experience that suggests a significant hazard, contraindication, side effect or precaution. It is any AE that at any dose fulfills at least one of the following criteria:

- is fatal; [results in death] [NOTE: death is an outcome, not an event]
- is life-threatening [NOTE: the term "life-threatening" refers to an event in which the patient was at immediate risk of death at the time of the event; it does not refer to an event which could hypothetically have caused a death had it been more severe].
- required in-patient hospitalization or prolongation of existing hospitalization;
- results in persistent or significant disability/incapacity;
- is a congenital anomaly/birth defect;
- is medically significant or requires intervention to prevent one or other of the outcomes listed above

Medical and scientific judgment should be exercised in deciding whether expedited reporting to the sponsor is appropriate in other situations, such as important medical events that may not be immediately life-threatening or result in death or hospitalization but may jeopardize the patient or may require intervention to prevent one of the outcomes listed in the definitions above. These situations should also usually be considered serious.

Examples of such events are intensive treatment in an emergency room or at home for allergic bronchospasm; blood dyscrasias or convulsions that do not result in hospitalization; or development of drug dependency or drug abuse.

An unexpected AE is one, the nature or severity of which is not consistent with the applicable product information.

Causality is initially assessed by the investigator. For Serious Adverse Events, possible causes of the event are indicated by selecting one or more options. (Check all that apply)

– Pre-existing/Underlying disease - specify

– Study treatment – specify the drug(s) related to the event

– Other treatment (concomitant or previous) – specify

– Protocol-related procedure

– Other (e.g. accident, new or intercurrent illness) - specify

The term severe is a measure of intensity, thus a severe AE is not necessarily serious. For example, nausea of several hours duration may be rated as severe, but may not be clinically serious.

A serious adverse event occurring during the study or which comes to the attention of the investigator within 15 days after stopping the treatment or during the protocol defined follow-up period, if this is longer, whether considered treatment-related or not, must be reported. In addition, a serious adverse event that occurs after this time, if considered related to test “drug”, should be reported.

Such preliminary reports will be followed by detailed descriptions later which will include copies of hospital case reports, autopsy reports and other documents when requested and applicable.

For serious adverse events, the following must be assessed and recorded on the AE form of the eCRF: intensity, relationship to test substance, action taken, and outcome to date.

The investigator must notify the Ethics Review Committee/Institutional Review Board of a serious adverse event in writing as soon as is practical and in accordance with international and local laws and regulations.

Appendix 4

Fibrosis Scores (HAI (Ishak) original and modified)

Score	Original HAI	Modified HAI
0	None	None
1	Mild piecemeal necrosis	Mild (focal, few portal areas)
2		Mild/Moderate (focal, most portal areas)
3	Moderate piecemeal necrosis (involves less than 50% of the circumference of most portal tracts)	Moderate (continuous around <50% of tracts or septae)
4	Marked piecemeal necrosis (involves more than 50% of the circumference of most portal tracts)	Severe (continuous around >50% of tracts or septae)
[a] The Periportal component of the Knodell HAI has been split into a Periportal piecemeal necrosis and a bridging/confluent necrosis component for better comparison to the other scoring systems. In order to recreate the original scale, the bridging/confluent necrosis component should be added to the Periportal piecemeal necrosis component.		

Appendices 8.2

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