Study of the frequency and distribution of IL 28B polymorphisms in hepatitis C virus infected patients and their association with virological markers and treatment response

Dissertation submitted in partial fulfilment of the rules and regulations for the M.D. (Branch-IV Microbiology) examination of the Tamilnadu Dr. M.G.R. Medical University to be held in April, 2015

CERTIFICATE

This is to certify that the dissertation entitled "Study of the frequency and distribution of IL28 B polymorphisms in hepatitis C virus infected patients and their association with virological markers and treatment response" is a bonafide work done by Dr. Pragya Ranjan towards the M.D. (Branch-IV Microbiology) Degree examination of the Tamil Nadu Dr. M.G.R. Medical University, to be held in April 2015.

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DECLARATION

I hereby declare that this M.D Dissertation entitled "Study of the frequency and distribution of IL 28B polymorphisms in hepatitis C virus infected patients and their association with virological markers and treatment response" is the bonafide work done by me under the guidance of Dr. Priya Abraham, Professor, Department of Clinical Virology, Christian Medical College, Vellore. This work has not been submitted to any other university in part or full.

Dr Pragya Ranjan

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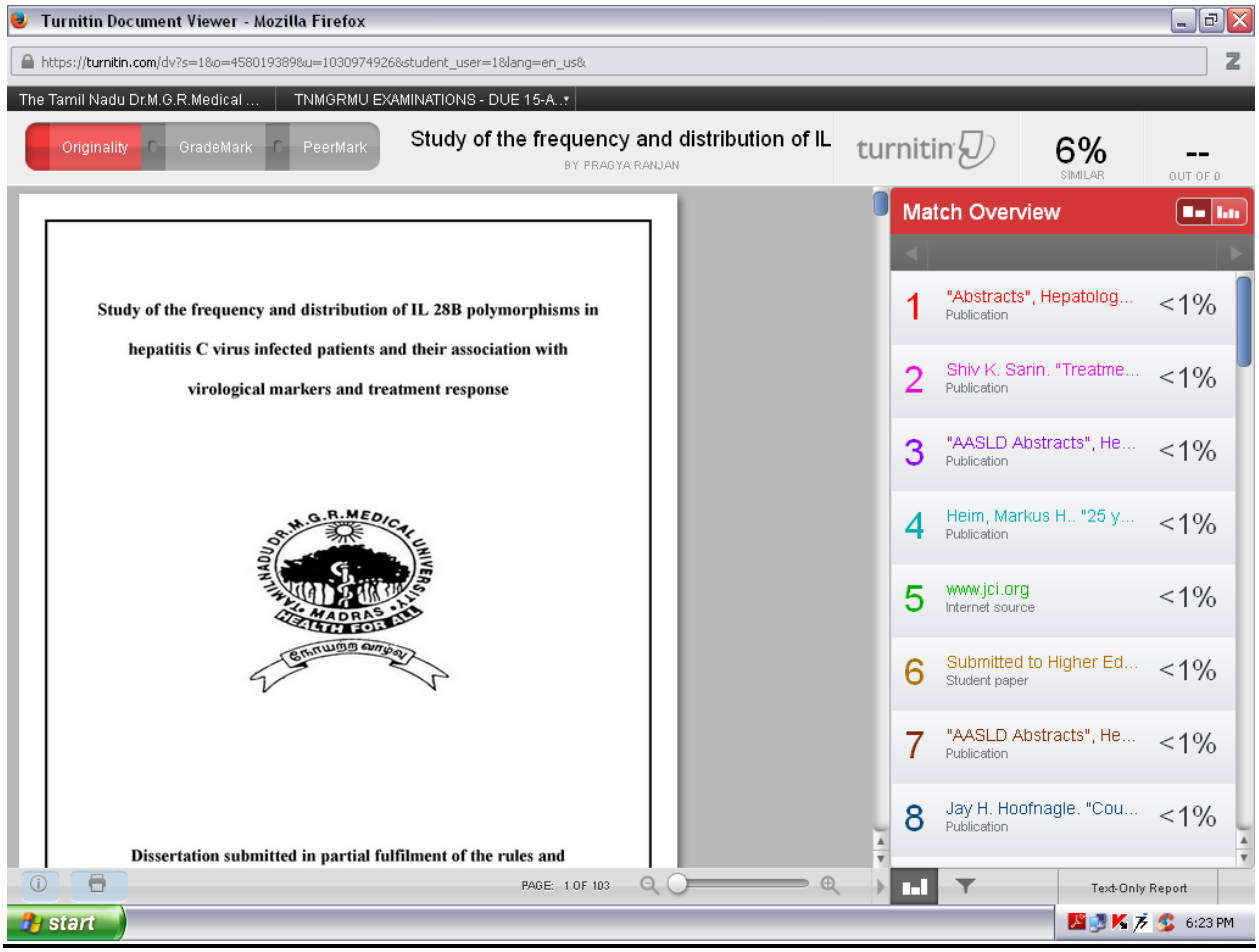
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I take great pleasure to express my sincere gratitude and thanks to all those people who have helped me and contributed in making this dissertation possible.

First and foremost, I express my profound gratitude to Dr Priya Abraham, my guide and mentor for her constant guidance, support and encouragement during the course of the study. She has been a source of inspiration to me throughout and I am grateful to her for the enriching and rewarding experience this dissertation turned out to be. Her utmost professional dedication is something I yearn to imbibe.

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- Most importantly, my family and my husband Manoj for being a constant source of love, concern, and strength.

Above all, God, who made all this possible

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Sub: **FLUID Research grant project NEW PROPOSAL:**
Study of the frequency and distribution of IL-28B polymorphisms in hepatitis C virus infected patients and their association with virological markers and treatment response.
Dr. Pragya Ranjan, Emp no: 20889, Clinical Microbiology, Dr. Priya Abraham, Clinical Virology, Dr. C E Eapen, Emp. No. 13779, Hepatology, Dr. Jeyamani R, Dr. Uday Zachariah, Dr. Ashish Goel, Dr. Sajith, Hepatology, Dr. John Fletcher, Clinical Virology, Ms. Jayashree Sivakumar, Emp. No. 32622, Clinical Virology, Dr. Jayaprakash Muliyil.

Ref: IRB Min No: 8202 dated 13.02.2013

Dear Dr. Pragya Ranjan,

I enclose the following documents:-

1. Institutional Review Board approval
2. Agreement

Could you please sign the agreement and send it to Dr. Nihal Thomas, Addl. Vice Principal (Research), so that the grant money can be released.

With best wishes,

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

Dr Nihal Thomas
MBBS MD MNAMS DNB (Endo) FRACP(Endo) FRCP(Edin)
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Ref: IRB Min No: 8202 dated 13.02.2013

Dear Dr. Pragya Ranjan,

The Institutional Review Board (Blue, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project entitled "Study of the frequency and distribution of IL-28B polymorphisms in hepatitis C virus infected patients and their association with virological markers and treatment response." on February 13, 2013.

The Committees reviewed the following documents:

1. Format for application to IRB submission
2. Proforma
3. Patient Information Sheet and Informed Consent Form (English, Tamil, Hindi, Telugu and Bengali)

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5. A CD containing documents1 – 4

The following Institutional Review Board (Research & Ethics Committee) members were present at the meeting held on February 13, 2013 in the CREST/SACN Conference Room, Christian Medical College, Bagayam, Vellore 632002.

Name	Qualification	Designation	Other Affiliations
Dr. Susanne Abraham	MBBS, MD	Professor, Dermatology, Venerlogy & Leprosy, CMC.	Internal, Clinician
Dr. Benjamin Perakath	MBBS, MS, FRCS	Professor, Surgery (Colorectal), CMC.	Internal, Clinician
Dr. Ranjith K Moorthy	MBBS MCh	Professor, Neurological Sciences, CMC	Internal, Clinician
Dr. P. Prasanna Samuel	B.Sc, M.Sc, PhD	Professor Dept. of Biostatistics, CMC	Internal, Statistician
Dr. Balamugesh	MBBS, MD(Int Med), DM, FCCP (USA)	Professor, Dept. of Pulmonary Medicine, CMC.	Internal, Clinician
Dr. Simon Rajaratnam	MBBS, MD, DNB (Endo), MNAMS (Endo), PhD (Endo), FRACP	Professor, Endocrinology, CMC	Internal, Clinician
Dr. Anup Ramachandran	PhD	The Wellcome Trust Research Laboratory Gastrointestinal Sciences	Internal
Dr. Chandrasingh	MS, MCH, DMB	Urology, CMC	Internal, Clinician

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Mr. Joseph Devaraj	BSc, BD	Chaplain, CMC	Internal, Social Scientist
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We approve the project to be conducted as presented.

The Institutional Ethics Committee expects to be informed about the progress of the project, any serious adverse events occurring in the course of the project, any changes in the protocol and the patient information/informed consent. And on completion of the study you are expected to submit a copy of the final report.

A sum of Rs. 60,000/- (Rupees Sixty Thousand only) will be granted for 18 months.

Yours sincerely

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

Dr Nihal Thomas
MBBS MD MNAMS DNB (Endo) FRACP(Endo) FRCP(Edin)
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INDEX

ABBREVIATIONS

ALT	Alanine aminotransferase
BMI	Body mass index
CLIA	Chemiluminescence immunoassay
DVR	Delayed virological response
DAA	Direct acting antiviral
ELISA	Enzyme-linked immunosorbent assay
EVR	Early viral response
ETR	End of treatment response
HCV	Hepatitis C virus
IFN	Interferon
ISGs	Interferon stimulated genes
IRF3	IFN regulatory factor 3
IPS-1	IFN-β promoter stimulator protein 1
IRES	Internal ribosomal entry site
ORF	Open reading frame
PAMPs	Pathogen-associated molecular patterns
PEG-IFN	Pegylated interferon
PCR	Polymerase chain reaction
RVR	Rapid viral response
RIBA	Recombinant immunoblot assay

RFLP	Restriction fragment length polymorphism
RIG-I	Retinoic acid inducible gene I
RT-PCR	Reverse-transcription polymerase chain reaction
RBV	Ribavirin
SNP	Single nucleotide polymorphism
SVR	Sustained viral response
TLR	Toll-like receptor
TMA	Transcription mediated amplification
UTR	Untranslated region

Abstract

Title of the abstract:

Study of the frequency and distribution of IL 28B polymorphisms in hepatitis C virus infected patients and their association with virological markers and treatment response

Department: Department of Clinical Microbiology

Name of the candidate: Dr. Pragya Ranjan

Degree and subject: M.D. Microbiology

Name of the guide: Dr. Priya Abraham

Keywords: Hepatitis C virus, IL 28B polymorphism, Interferon, Ribavirin, Sustained viral response, Rapid viral response

Objectives:

The objective of this study was to determine the frequency and distribution of IL 28B polymorphisms in hepatitis C virus infected patients and their impact on treatment response in genotype 1, 3 and 4 infections. We also evaluated the association of other host and viral factors with sustained virological response.

Methods:

Fifty seven hepatitis C virus infected patients (genotype 1=12, 3=43 and 4=2) on treatment with interferon (standard/pegylated) and ribavirin were recruited. DNA was analyzed for the IL 28B polymorphisms using PCR- RFLP (CC, CT and TT for rs12979860 and TT, GT and GG for rs8099917). Bidirectional sequencing was performed on a subset of samples for verification of PCR-RFLP results. Information on age, weight, height, diabetic status, pre-treatment viral load and alanine aminotransferase levels was obtained from clinical records.

Results:

The frequency distribution of rs12979860 CC/CT/TT genotypes was found to be 60%, 33% and 7% respectively. For rs8099917 genotype, the TT/GT/GG distribution was 72%, 23% and 5% respectively. Of the 57 patients recruited, 34 completed follow up during the course of the study. Sustained viral response was seen in 56% of these cases (57% in genotype 1 and 54% in genotype 3). The CC genotype at rs12979860 loci was found to be associated with sustained viral response (P value=0.012) and rapid viral response (P value=0.017). No association was found between rs8099917 polymorphism and treatment response.

Age, gender, body mass index, diabetic state, baseline viral loads, pre-treatment alanine aminotransferase levels and treatment modality were not found to be associated with sustained viral response. Rapid viral response was found to be predictive of sustained viral response (P value=0.005).

Conclusion:

The CC genotype at rs12979860 loci was found to be associated with sustained viral response and rapid viral response.

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INTRODUCTION

Hepatitis C virus (HCV) is an enveloped, single stranded RNA virus belonging to the family *Flaviviridae*. It is a common cause of post transfusion hepatitis in the resource poor settings. HCV infection is a global health problem with a worldwide prevalence of around 2-3% (1), with more than 185 million seropositive people worldwide (2). India has over 10 million HCV seropositive individuals (3), the disease being largely spread by blood transfusion and unsafe injection practices (4). Spontaneous resolution occurs in about 15 to 40% of acutely infected individuals while in the rest chronic infection is established. Chronic hepatitis C shows a variable clinical outcome ranging from chronic hepatitis, liver cirrhosis, end-stage liver failure, and occasionally hepatocellular carcinoma, which is dependent on an array of host and viral factors (5).

Genomic heterogeneity has led to the classification of HCV into various genotypes and subtypes (6). HCV genotypes do not differ in transmissibility or level of replication but are largely different in their response to interferon-based therapies, thus impacting the duration of treatment needed. There is a huge geographic variation in the distribution and prevalence of HCV genotypes globally. The predominant genotype in the western hemisphere is genotype 1, whereas genotype 3 is the commonest in the Indian subcontinent, followed by 1 and 4 in that order. The largest study from this country (7) found that genotype 3 accounted for 64% of all HCV infections , followed by genotype 1 which was 25%.

Detection of antibodies against HCV indicates exposure to the virus. Viral load testing is necessary to establish the presence of active infection. Treatment becomes crucial keeping in view the chronic nature of the infection and accompanying complications. Antiviral therapy

helps in preventing both hepatic as well as extrahepatic sequelae of infection. Currently, pegylated interferon (PEG-IFN) plus ribavirin (RBV) is the standard of care therapy for chronic hepatitis C, administered for either 48 weeks (genotypes 1 and 4) or 24 weeks (genotypes 2, 3, 5 and 6). However, treatment is expensive and is associated with significant adverse effects, which may be severe enough to lead to premature discontinuation of treatment. This necessitates serial monitoring of viral load in patients on therapy to assess and prognosticate the treatment response. The recommended time points of monitoring are:

Rapid viral response (RVR) at 4 weeks

Early viral response (EVR) at 12 weeks

End of treatment response (ETR) at 24/48 weeks, based on genotype

Sustained viral response (SVR) at 24 weeks after ETR

SVR rates of 40–50% are seen with genotype 1 HCV, and upto 80% in genotypes 2 and 3 infections (8).

Since treatment is expensive and often accompanied by several adverse effects, the significance of viral and host factors which impact on severity of disease and response to treatment becomes immense. Age<40 years, female gender, Caucasian race, body weight <85 kgs, absence of diabetes mellitus, absence of steatosis on liver biopsy, fibrosis score on liver biopsy ≤2 are established factors which predict a good response to therapy (9).

Recently several genome wide association studies have shown that single nucleotide polymorphisms (SNPs) within or adjacent to the IL28B gene (rs12979860 and rs8099917) are strongly associated with response to PEG-IFN/RBV therapy in genotype 1 infections (10–12). The IL28B gene located on chromosome 19 codes for Interferon λ3 which induces antiviral and anti proliferative activity in many cell types and upregulates interferon stimulated genes

(ISGs) (13). The possible genotypes at rs12979860 are CC, CT and TT, while those at rs8099917 are TT, TG and GG. The CC genotype of rs12979860 and TT of rs8099917 have been shown to be associated with a better treatment response. These polymorphisms show a marked differential racial distribution (10) explaining much of the observed differences in the response rates to treatment in different ethnicities. Its association with spontaneous clearance of HCV infection has been shown irrespective of the viral genotype (14). The association with virological response has also been found in HCV genotype 4 (15). The association of IL28B polymorphisms with response to treatment in HCV genotype 2 and 3 infections have remained controversial. Studies have shown conflicting results. However, the largest meta analysis by Jiménez-Sousa *et al.* found significant associations of rs12979860 and rs8099917 polymorphisms with treatment response in genotypes 2 and 3 infected patients, but the strength of association was three fold lower than that for genotypes 1 and 4 (16).

In 2012, Sivaprasad *et al.* (17) studied the distribution of genotype and allelic frequency of IL28B rs12979860 polymorphism in 220 healthy uninfected controls in Andhra Pradesh, India, and found that the frequency of CC genotype (59%) was significantly higher compared to CT (34.09%) and TT (6.81%). Thereafter, Gupta and colleagues (18) from New Delhi analysed the rs12979860 SNP in 356 patients infected with HCV genotype 3 and found the CC genotype to be an independent strong predictor of RVR and SVR. Another group from Kolkata (19) has found genotypes CC at rs 12979860 and TT at rs8099917 to be strongly associated with SVR in their study on 83 HCV genotype 3 patients. However, association of these polymorphisms with genotype 1 has not been looked at in both the studies.

This study aims to determine the frequency and distribution of IL28B gene polymorphisms in patients with chronic HCV infection harbouring genotype 1 in addition to 3, and to study the

association of these SNPs with response to IFN based treatment. It would also study the correlation of other host factors like age, gender, body mass index, diabetes and baseline alanine aminotransferase (ALT) levels with treatment response.

AIM

To study the frequency and distribution of IL28B polymorphisms in hepatitis C virus infected patients and their association with virological markers and treatment response.

OBJECTIVES

1. To study the frequency and distribution of IL28B polymorphisms in hepatitis C virus infected patients.

2. To study and compare sustained viral response rates in hepatitis C virus genotype 1 and 3 infections.

3. To study the association of IL28B polymorphisms with sustained viral response after treatment in hepatitis C virus genotype 1 and 3 infected patients.

4. To study the association of other factors like age, gender, body mass index, diabetes, pre-treatment viral loads, baseline alanine aminotransferase levels and treatment modality with sustained virological response.

5. To study the association of IL28B polymorphisms with virological response during the course of treatment (Rapid viral response, Early viral response, End of treatment response).

REVIEW OF LITERATURE

Ever since its discovery in 1989 as the causative agent of transfusion associated *non-A non-B hepatitis,* HCV has been increasingly recognized as a global health concern. First thought to be a trivial infection limited to the intravenous drug users and blood product recipients in developed countries, it is now established as the predominant cause of post transfusion hepatitis and chronic liver disease worldwide, more so in the developing parts of the world. Owing to the tendency of HCV to cause persistent infection, it is associated with a wide disease spectrum ranging from chronic hepatitis, liver cirrhosis, end-stage liver failure, and occasionally hepatocellular carcinoma.

1. Epidemiology

1.1 Global burden:

HCV infection has a worldwide distribution, affecting persons of all ages, races, genders and regions of the world. The global prevalence of the infection is estimated to be 2-3% (1), with more than 185 million seropositive people worldwide (2). HCV accounts for more than 350,000 deaths annually, most of which are attributable to liver cirrhosis and hepatocellular carcinoma (20). Prevalence higher than the global average has been reported from Africa (3.2%) and the Middle East (4.7%) (1).

1.2 Indian Scenario:

HCV infection is an important emerging cause of liver disease in India. Blood transfusion and unsafe injection practices are believed to be two major routes of spread of the virus in our part of the world (4). There is a dearth of large community based studies to estimate the real burden of

the infection in India, however, in the largest such study by Chowdhury and colleagues from West Bengal (21), the prevalence of HCV antibody was found to be 0.87% (26 of 2973 samples). HCV RNA was detected in 81% of those who were anti-HCV positive. With our teeming population, this would translate to more than 10 million HCV seropositive individuals across the nation (3), of whom 8 million may be viraemic.

A study on a rural population in Maharashtra (n=1054) found a very low prevalence of 0.09% (22), whereas two studies from Andhra Pradesh found the prevalence to be 1.4% and 2.02% respectively (23,24).

Seroprevalence in voluntary or replacement blood donors has been found to range from 0.7% to 1.8% (25). What is worrisome is the very high prevalence of 55.3% and 87.3% in professional donors as per two studies in western India (26,27). High prevalence has also been reported from other high risk groups for the infection; 16.7% to 21% among thalassemia patients, 23.9% among multiply transfused hemophilia patients, 9.93% in hemodialysis patients and 92% in intravenous drug users in the Northeast (3). All of this emphasizes the need for stringent blood banking and injection practices throughout the nation.

2. Hepatitis C Virus

2.1 Classification and Taxonomy:

Owing to its structure, genomic organisation and replication, HCV has been classified as a member of the family *Flaviviridae*, along with other related positive-stranded RNA viruses. The virus, however, is distinct enough to merit classification within a separate genus, *Hepacivirus*, which gets its name from the Greek word ‘hepatos’ meaning liver . The other two genera within *Flaviviridae*, genus Flavivirus (e.g., Japanese encephalitis virus, dengue viruses and yellow

fever virus) and genus Pestivirus (e.g., bovine viral diarrhoea virus and classical swine fever virus), differ from HCV in the organization of certain structural proteins (28).

2.2 Structure of the virus:

HCV is an enveloped, 9.6 kilobases long positive sense single-stranded RNA virus (28). Like the other members of family *Flaviviridae*, its genome has one large open reading frame (ORF) which accounts for over 95% of the sequence. The ORF encodes a single large polyprotein, about 3010 amino acids long, which undergoes post-translational modifications to yield various viral proteins. Flanking the ORF at both 5´- and 3´- ends are highly conserved untranslated regions (UTRs), which mediate crucial steps in viral replication.

2.2.1 Untranslated regions

About 341 nucleotides long, the 5′UTR is a highly conserved region (29). It has an approximately 300-nucleotide long segment, known as "internal ribosomal entry site"(IRES), that mediates direct binding of the 40S host ribosome subunit to the viral genome and facilitates the process of translation (30).

The 3′UTR contains a 40 nucleotide long variable region, a poly U/UC tract of heterogeneous length, and a highly conserved 98-nucleotide long sequence, designated the X tail or 3′X(31). Parts of this 3′X tail form a "kissing loop" interaction with the NS5B coding region, which along with a 33 consecutive U residue segment in the poly-U/UC tract, is absolutely necessary for viral RNA replication (32).

2.2.2 Polyprotein

The ORF encodes a polyprotein that is processed into 10 proteins. The polyprotein can be functionally divided into three segments.

A. The NH_2-terminal region, comprising the structural proteins (core and the envelope glycoproteins, E1 and E2)
B. The central region including two proteins (p7 and NS2) which are essential for virion production but are not required for viral RNA replication; and
C. The COOH-terminal region, which consists of five nonstructural proteins (NS3, NS4A, NS4B, NS5A, and NS5B) that are needed for RNA replication.

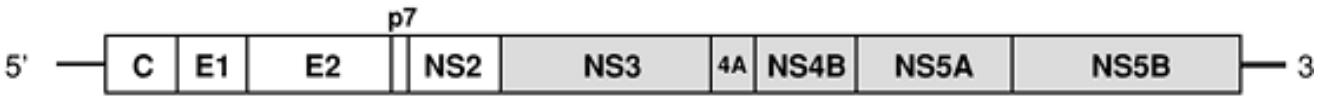


Figure 1.Organization of the HCV genome and polyprotein

2.2.2.1 Structural proteins

First product of the polyprotein is the highly basic core protein, C, which binds with RNA to form the nucleocapsid (33). Next two domains in the polyprotein are processed into two glycoproteins, E1 and E2. These transmembrane proteins are essential for initial viral attachment to host cells, thus facilitating cell entry at specific steps (34). E2 contains a hypervariable region, whose rapid evolution during the course of an infection prevents recognition by antibodies (35).

2.2.2.2 p7 and NS2 proteins

These two proteins do not play a role in RNA replication but are crucial in virion morphogenesis and release. p7 (formerly NS2A) functions as a viroporin, transporting calcium ions from endoplasmic reticulum into the cytoplasm, thereby representing a possible therapeutic target (36).

The NS2 (formerly NS2B) protein has a protease domain that mediates cleavage at the NS2/NS3 junction, essential for the production of infectious virions(37).

2.2.2.3 Nonstructural proteins

All the five nonstructural proteins (NS3, NS4A, NS4B, NS5A, and NS5B) are involved in RNA replication. The amino-terminal of the NS3 protein possesses serine protease activity and carboxy-terminal has RNA helicase activity. The protease is responsible for cleavage of the NS3/4A, NS4A/4B, NS4B/5A, and NS5A/5B junctions during processing of the polyprotein. The NS4A protein functions as a cofactor for the NS3 protease. NS4B is thought to play an important role in modifications of endoplasmic reticulum membrane, and thus in the organisation of the membrane-bound replication complex. A part of the NS5A phosphoprotein, known as interferon sensitivity determining region (ISDR), is believed to determine response to IFN based therapy. NS5B encodes a viral RNA-dependent RNA polymerase (RdRp), which is a key component for HCV replication (38).

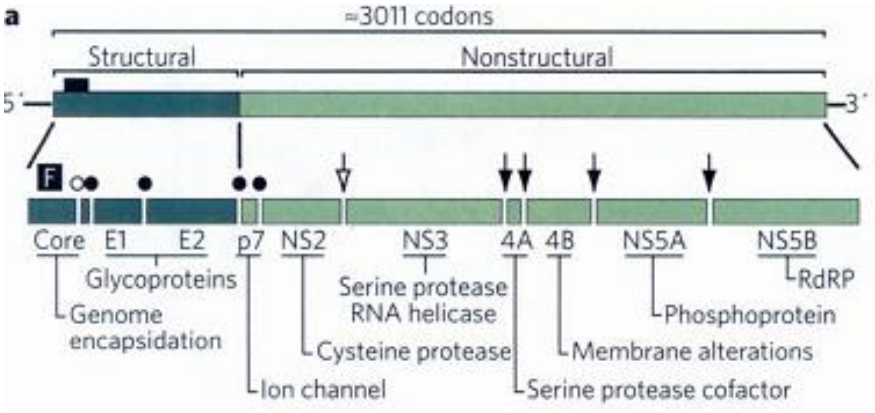


Figure 2.Genomic structure of hepatitis C virus, Adapted from Lindenbach and Rice (38)

2.3 Replication:

Life cycle of HCV begins with attachment and internalization of the virus into the host cell, which is mediated by viral envelope glycoproteins E1 and E2. A number of host cellular receptors such as CD81, DC-SIGN, SR-BI claudin-1, and occludin are believed to be necessary for this process. After attachment and entry, uncoating of the nucelocapsid occurs, leading to release of the viral RNA into host cytoplasm. Being positive stranded, HCV RNA acts as messenger RNA (mRNA) and translation of the polyprotein is initiated following ribosomal binding mediated by the HCV IRES domain. This is followed by a number of cleavages of the polyprotein by both cellular and viral proteases, resulting in the production of various structural and non-structural proteins, as outlined in the previous section. Following cleavage, the core protein stays in cytoplasm, while E1 and E2 are secreted into lumen of endoplasmic reticulum. The non structural proteins assemble to form a membrane-bound replication complex, where the viral NS5B RdRp facilitates the synthesis of a negative-stranded RNA intermediate. This subsequently serves as a template for synthesis of positive-stranded genomic RNA. Following this, RNA, along with the core, E1 and E2 proteins gets packaged into

new viral particles. After maturation and assembly, newly produced virions are released from the host cell through the secretory pathway.

2.4 Genetic diversity:

2.4.1 Quasispecies variation

The replication process of HCV is highly prone to errors and mutations due to its rapidity and lack of proof-reading by the NS5B RNA polymerase. This, coupled with immunologic selection, leads to accumulation of a multitude of closely related but distinct HCV variants within an infected individual, known as a quasispecies (39). This heterogeneity of the viral population may rapidly select treatment-resistant clones, thus possibly reducing treatment efficiency of the new direct acting antiviral (DAA) drugs recently approved for treating HCV infection (40).

2.4.2 HCV genotypes

In addition to quasispecies variation that occurs in a single infected individual, there is also tremendous heterogeneity among sequences of HCV isolates from different individuals. This has led to their classification into genotypes and subtypes. Based on sequence homologies, phylogenetic studies have shown that there are seven genotypes categorized 1 through 7 and 67 confirmed subtypes named with the letters a, b, c and so on following the genotype Genotype 7 was long considered a provisional genotype represented by a single strain, but has now been confirmed as a separate genotype (6). Genotyping is usually done by sequencing either the 5′UTR/core, NS3 or of the NS5b region of HCV genome. Across genotypes, the diversity at the nucleotide level is estimated to be about 30% (35,41). Within individual genotypes, subtypes differ by at least 15% in nucleotide sequence identity within the core/E1 and NS5B regions (6). HCV genotypes do not differ in transmissibility or level of replication but are largely

different in their response to interferon-based therapies, thus impacting the duration of treatment needed.

2.4.2.1 Global distribution of HCV genotypes

The geographical distribution of different genotypes is quite distinct. Genotype 1 is found to be the commonest genotype worldwide with a wide distribution in USA and northern Europe (35,41). Genotypes 2 and 3 are also found worldwide, with a higher prevalence in Europe, North America, and Japan (42). HCV genotype 3 infection is endemic in Southeast Asia and the Indian subcontinent. It is also particularly prevalent in intravenous drug users in the USA and Europe. Genotype 4 infections are mainly prevalent in North Africa and Middle East. Genotype 5 appears to be confined to South Africa and genotype 6 to intravenous drug users in Southeast Asia and more recently in Australia. Genotype 7 has been reported from a single case in central Africa. (35,41,43)

2.4.2.2 Distribution of HCV genotypes in India

There are a few studies which have attempted to establish the distribution of HCV genotypes in the country. In the largest such study by Christdas *et al.*(7), spanning over a decade (2002-2012) and including 451 patients from various parts of the Indian subcontinent, genotype 3 was found to be the most predominant (63.85%), followed by genotype 1, 4 and 6 (25.72%, 7.5% and 2.7% respectively). Genotype 2 was found in only one patient from Northeast India, and genotype 5 in none. (As is mentioned in the table below, genotype 2 has been infrequently reported by other authors, while genotype 5 is yet to found in our part of the world.) Genotype 1 was commoner in South India, while genotype 3 was more prevalent in Eastern and Northeastern parts of the country. Genotypes 4 and 6 appeared to be restricted geographically to the Southern and North-

Eastern parts of the country respectively, which has been published previously as well (44,45). Recombinant strains of genotype 1 and 2 were isolated from two patients.

In another study on 398 patients from North and Central India (46), the findings were similar. Genotype 3 was the commonest genotype, seen in 80.2% patients, followed by genotype 1 in 13.1% patients. Genotypes 4 (3%) and 2 (2.5%) were rare. There were no cases of genotype 5 and 6 infections. Five patients showed infection with mixed genotypes.

The following table summarizes the various studies estimating the distribution of HCV genotypes throughout the country.

Table 1.Distribution of HCV genotypes in India

Author	**Year**	**N**	**Distribution of genotypes (in %)**					
			1	2	3	4	6	Misc.
Christdas *et al.*(7)	2013	451	25.7	0.002	63.9	7.5	2.7	0.004[#]
Chakravarty *et al.*(47)	2013	31	29	9.6	61.2	-	-	-
Chakravarty *et al.*(48)	2011	71	31	5.6	63.4	-	-	-
Hissar *et al.*(46)	2006	398	13.1	2.5	80.2	3	-	1.3[#]
Chaudhuri *et al.*(49)	2005	420	10.2	3.8	79.8	-	-	6.2[#*]
Singh *et al.*(50)	2004	36	13.8	5.5	66.6	2.7	-	11.1[*]
Raghuraman *et al.*(51)	2003	90	18.9	1.1	62.2	5.6	-	12.2[*]
Chandra *et al.*(23)	2003	18	66.7	-	33.3	-	-	-
Das *et al.*(52)	2002	153	24.2	2	69.9	3.9	-	-
Amarapurkar *et al.*(53)	2001	61	21	25	54	-	-	-
Valliammai *et al.*(54)	1995	24	87.5	-	12.5	-	-	-

Genotype 5 has not been reported in any of these studies.

mixed infection * untypeable infection

3. Natural history of the disease

3.1 Acute Hepatitis C and spontaneous clearance

HCV can cause both acute and chronic hepatitis. Acute infection with HCV is mostly asymptomatic. HCV RNA is detectable in majority of patients within 1-2 weeks and is followed by a rise in serum transaminases by 2-8 weeks. About 25 to 30% of patients with acute HCV infection develop symptoms within 3-12 weeks of exposure to the virus (average 7 weeks). Anti-HCV seroconversion occurs near the onset of symptoms. However anti-HCV is unreliable in the diagnosis of acute HCV infection as up to 30% of patients will test negative at the onset of symptoms because of delayed seroconversion. Almost all patients will eventually develop anti-HCV, though titres may be low in the context of immunosuppression (55).

An estimated 15-40% patients spontaneously clear the virus, becoming HCV RNA negative, while majority infected with HCV will go on to develop chronic infection.

3.2 Chronic Hepatitis C and progression of fibrosis

Persistence of HCV RNA for more than 6 months after onset of infection defines chronic hepatitis C. Age at acquisition of infection, sex, race, immune status of the patient, co-infections, along with other host and viral factors influence chronicity of the infection (5). The early phase of the infection is marked by appearance of HCV RNA, followed by rise in serum transaminases. It must be noted that in the time period of evolution from acute to chronic hepatitis, HCV RNA and enzyme levels can fluctuate remarkably. Once the infection gets persistent, viral load tends to stabilize. Spontaneous resolution of chronic infection is unusual. Fatigue, abdominal discomfort, nausea, and poor appetite are the most common symptoms seen (55). The disease may remain clinically silent for decades. However, hepatocellular inflammation and fibrosis

continues, leading to progressive liver disease. The rate of progression of the disease is again determined by a multitude of modifiable and non modifiable factors. Progressive hepatic fibrosis may lead to cirrhosis and decompensated liver disease. Such patients are at highly increased risk of hepatocellular carcinoma, with 1 to 4% of patients developing this complication each year (56). It usually takes more than two decades of infection for these long term complications to develop, unless accelerated by coexistent factors.

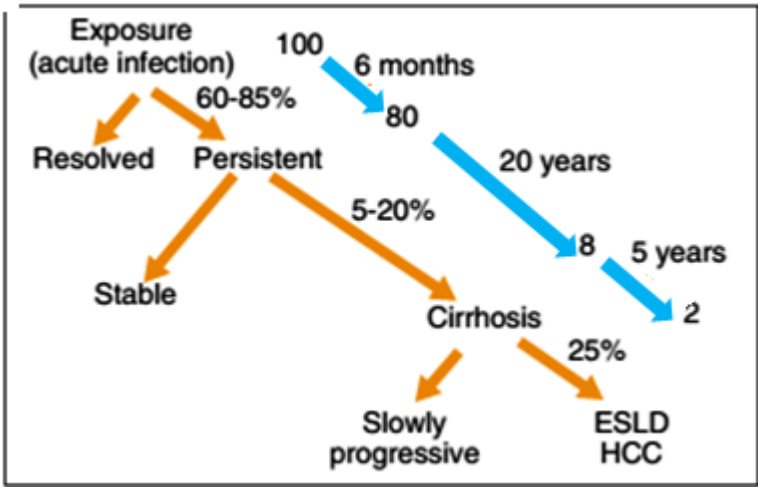


Figure 3. Natural history of HCV infection

Adapted from Mandell, Douglas and Bennett's Principles and Practice of Infectious Disease, 7th edition

4. Immune Response to HCV Infection

Viral infection triggers an array of intracellular events that lead to the development of an antiviral state in the infected cell and the surrounding tissue. After viral entry into the host, pathogen-associated molecular patterns (PAMPs) in the viral genome are recognised by PAMP receptors expressed on the host cell, initiating the host immune response. Retinoic acid inducible gene I (RIG-I) and Toll-like receptor 3 (TLR3) are two major receptor pathways triggered by

HCV RNA. This subsequently stimulates interferon stimulated genes (ISGs) inducing endogenous interferon (IFN) production, and thus building the initial antiviral defence (57). For successful replication and establishment of a persistent infection, HCV develops various strategies to evade host immune response. It is the balance between the two which determines progression of the disease.

4.1 Innate Immune response

4.1.1 Interferons and Interferon Stimulated Genes

The first response to HCV infection is by the production of endogenous IFN by the infected hepatocytes. This begins with TLR-3 and RIG-1 mediated sensing of HCV RNA, which through various mediators leads to signalling of IFN regulatory factor 3 (IRF3). This induces the transcription of IFN-β, creating an antiviral state in infected and uninfected neighbouring cells, via paracrine effects, limiting cell to cell spread (58). IFN-β binds to the IFN-α/β receptor, activating the JAK/STAT pathway. This results in induction of IFN stimulated genes (ISGs), which have different antiviral properties, such as degradation of viral RNA, inhibition of translation and destabilisation of secondary structures of viral RNA. Some pattern recognition and signalling molecules like RIG-I are also ISGs, whose levels markedly increase from low basal levels, increasing the sensitivity of downstream signalling in infected tissues, and promoting IFN and ISG production. Another ISG, IRF7 stimulates IFN-α production, thus diversifying the IFN response, and providing a positive feedback to ISG expression (57,59). The current treatment for HCV capitalises on the IFN- α component of immune response.

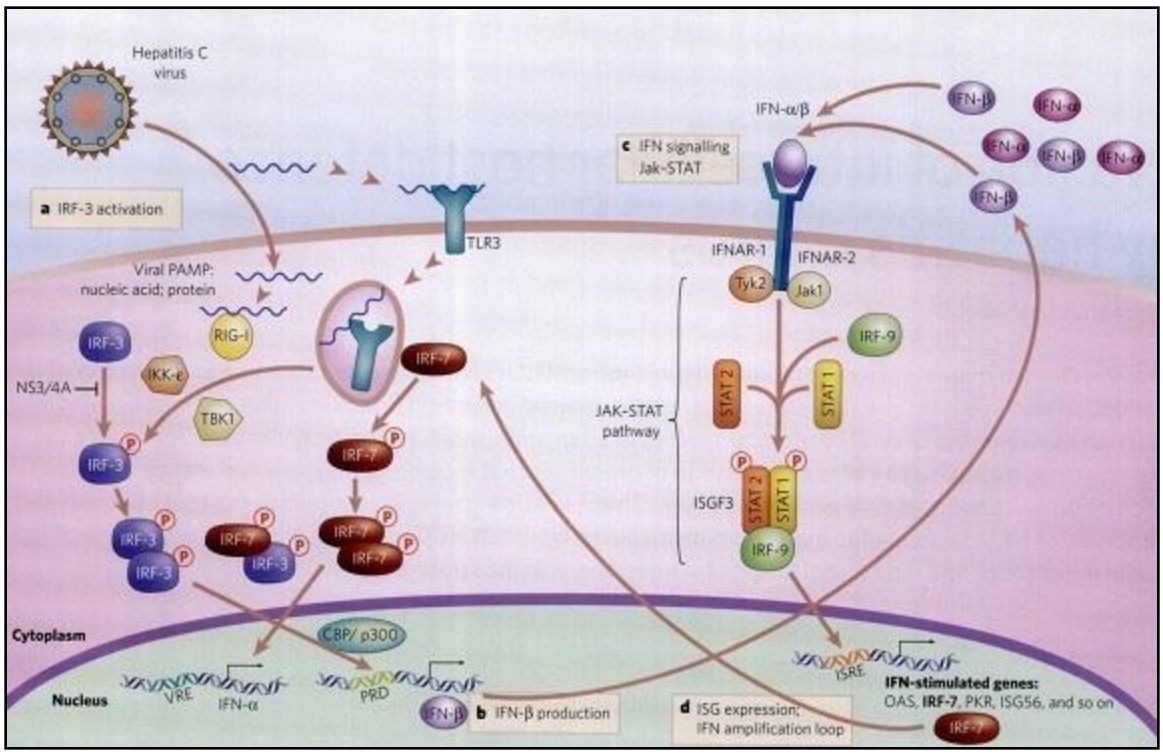


Figure 4.Molecular processes that signal the host response during HCV infection, adapted from Gale and Foy (57)

4.1.2 Attenuation of Innate Immune Response by HCV

HCV is known to employ multiple strategies to attenuate innate IFN response.

1. The HCV NS3/4A protein, via its protease activity, cleaves two important host adapter molecules TRIF and IFN-β promoter stimulator protein 1 (IPS-1), thereby blocking TLR3 and RIG-I signalling and hence IRF3 activation.
2. HCV core protein brings about impairment of JAK/STAT signalling pathway and ISG expression.

3. HCV NS5A stimulates IL-8 production which inteferes with ISG expression, thus antagonizing type I IFN signalling.

Other than the aforementioned mechanisms, HCV also interferes with functioning of dendritic cells and NK cells, both of which contribute to defence against the virus (59).

4.2 Adaptive Immune Response

4.2.1 Humoral Immunity

Antibodies corresponding to structural and non structural proteins of HCV are detectable in about 7 to 8 weeks of infection. These antibodies are neutralizing in nature, differing in their breadth and mechanisms of neutralization. The antibodies are isolate specific, and together with $CD8^+$ T cells contribute to the evolution of HCV quasispecies by exerting selection pressure. Lack of temporal relation of these antibodies to viral recovery and demonstration of HCV clearance in individuals with agammaglobulinemia led to the belief that humoral immune response was neither necessary nor sufficient for viral clearance (59,60).

However, recent studies have elucidated the role of the neutralizing antibodies in disease outcome. Early and rapid induction of these antibodies has been found to lead to spontaneous resolution of infection, contrary to the cases of chronic infection, where antibodies were either absent or very low in titre in early phase of the infection, thus suggesting a crucial role in the outcome of the infection (61).

4.2.2 Cellular Immunity

HCV specific $CD8^+$ and $CD4^+$ T cell response is known to be critical for HCV clearance. A functional $CD4^+$ response is an important factor dictating the fate of HCV infection by

production of IL-2 and IFN-γ. Vigorous proliferation of HCV specific $CD4^+$ T cells is seen in individuals who clear the virus, in contrast to an impaired or weak response in those who progress to chronic disease (62).

On the other hand HCV specific $CD8^+$ cells are detectable in cases of acute infection irrespective of virological outcome. In acute infection some $CD8^+$ cells show a "stunned" phenotype and are unable to produce IFN-γ. However, as $CD4^+$ T cell responses develop and viremia declines, this dysfunction resolves and memory cells become detectable (63).

In cases of recovery, durable populations of memory T cells are seen. In chronic infections, persistent antigenic stimulation along with impaired $CD4^+$ T cell function leads to $CD8^+$T cell exhaustion. This state is marked by loss of $CD8^+$ T cell cytotoxic functions, TNF-α production, and eventually IFN-γ production along with dysfunctional memory T cells as is often the case in chronic HCV infection (59).

4.2.3 Evasion of Adaptive Immune Response by HCV

A lot of theories for persistence of HCV infection are hypothesized, but the following three mechanisms have substantial experimental support (60).

1. Mutational escape of viral epitopes

   The error prone nature of the viral polymerase generates viral variants capable of evading cytotoxic T cells and neutralizing antibodies.
2. Functional anergy of $CD8^+$ T cells

   As discussed in the previous section, HCV specific $CD8^+$ T cells may be anergic or functionally impaired in chronic infections.
3. Regulatory T cell populations

Intrahepatic $CD8^+$ T cell populations producing IL-10 are known to occur in chronic infections. IL-10 impairs production of IFN-α and downregulates effector T cell responses.

The outcome of HCV infection is depicted in the flowchart below.

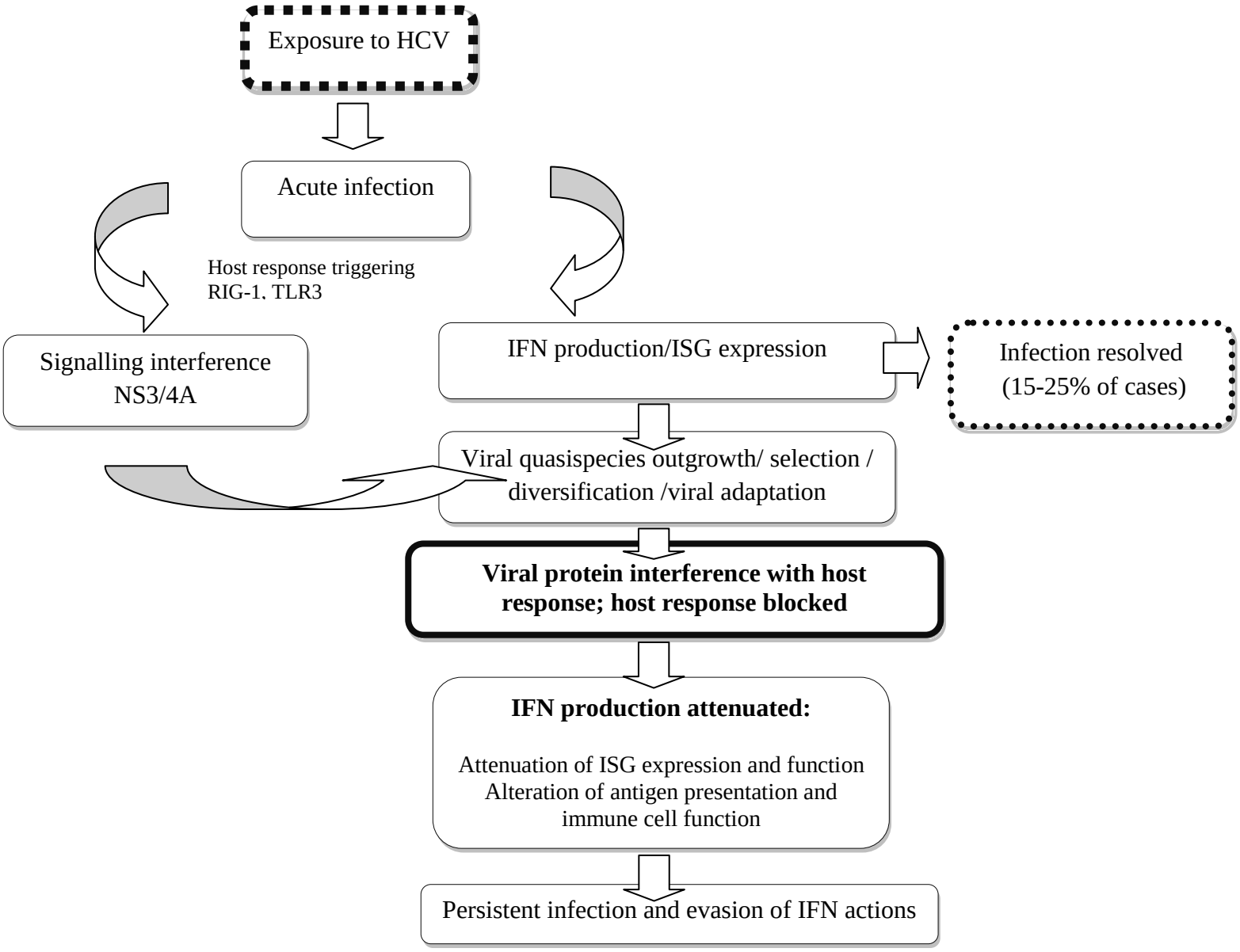


Figure 5.Outcome of HCV infection, Adapted from Gale and Foy (57)

RIG-1: Retinoic acid inducible gene I TLR3: Toll-like receptor 3

IFN: Interferon ISG: Interferon stimulated genes

5. Diagnosis of HCV infection

Testing for HCV infection is mainly done for a clinical diagnosis of liver disease in symptomatic individuals and as a part of mandatory screening in blood banks for all donors. It is also advisable for individuals who are at a high risk for the infection. Guidelines recommend HCV screening in persons with HIV infection, haemophilia, haemodialysis, illicit drug use, recipients of blood transfusion or organ transplantation before 1992, children born to HCV infected mothers and health care workers after an exposure (64).

Diagnostic tests for HCV are broadly grouped into serologic assays to detect the presence of virus specific antibodies, and molecular tests for detection and quantification of viral RNA and genotyping of the virus.

5.1 Serology

Detection of HCV specific antibodies is an indicator of infection with the virus and not immunity. Immunoassays, based either on enzymatic reactions (Enzyme-linked immunosorbent assay, ELISA) or light emission (Chemiluminescence immunoassay, CLIA) are the standard tests used by most diagnostic laboratories. Different generations of HCV ELISA detecting antibodies to different recombinant polypeptides have been developed. While the first generation ELISA targeted a part of the NS4 region of HCV genome, the second generation included a protein derived from NS3 and a part of core (C-22) additionally. The third generation ELISA detects antibodies against NS5 as well, and has a high sensitivity of about 97%. Recombinant immunoblot assay (RIBA) can be used as a supplemental test to identify the specific antibodies against individual HCV antigens. Some rapid immunoassays have been developed as point of care tests for rapid detection of HCV antibody.

5.2 Molecular assays

5.2.1 Detection of viral nucleic acid

Detection of HCV RNA is necessary to establish active infection, either acute or chronic, as well as for monitoring the patients on treatment. Reverse transcription polymerase chain reaction (RT-PCR), real time RT-PCR, transcription mediated amplification (TMA), and branched DNA testing can be used. Assays that detect nucleic acids can be qualitative or quantitative. While qualitative methods may be sufficient for screening in blood banks, quantitative assays are used to measure the baseline viral load prior to initiation of therapy, and then at specified time points for monitoring of treatment response during the course of therapy. WHO has recommended the use of a standard "International Units" (IU) for measurement of the viral RNA instead of viral copies. Most contemporary assays have excellent specificities 98 to 99% and sensitivity varying from 10 to 50 IU/mL (64).

5.2.2 Viral genotyping

Determination of genotype of the infecting virus is necessary to tailor the duration of treatment needed, as well as to predict the probability of response. Genotyping can be done by sequencing either the 5′UTR/core, NS3 or the NS5b region of HCV genome. A number of assays are available for the same and include real time PCR with genotype specific probes and primers, reverse hybridization of PCR products onto genotype specific probes coated on solid supports (line probe assay), PCR-restriction fragment length polymorphism (RFLP), where the PCR products are digested with restriction enzymes, to obtain fragments of varying length depending upon the genotype.

5.3 IL28B genotyping

Single nucleotide polymorphisms in the region upstream of IL28B gene (rs12979860 and rs8099917) have been found to be strong predictors of response to interferon based therapy. These polymorphisms can be detected by PCR-RFLP, direct sequencing or pyrosequencing. These polymorphisms can be used to prognosticate treatment, but absence of tools to detect these polymorphisms, by no means, impacts the treatment.

6. Treatment of Hepatitis C

6.1 Rationale for treatment

Hepatitis C is a severe infection causing considerable morbidity and mortality globally. The main concern associated with the infection is progression to liver cirrhosis and its accompanying complications. Patients with chronic infection are at risk of extrahepatic manifestations even in the absence of progressive fibrosis, some of which may be severe. Antiviral treatment is necessary to prevent both the hepatic as well as extrahepatic sequelae of infection. Virologic cure, marked by sustained lack of viraemia six months after completion of therapy, is associated with lessening of liver inflammation, as evidenced by stabilized enzyme levels and decrease in the rate of progression of liver fibrosis. Timely treatment has been shown to decrease the development of end stage liver disease, need for liver transplantation, hepatocellular carcinoma rates and liver related mortality (65).

6.2 Drugs used for treatment

1) Interferon and Ribavirin:

For the past two decades, recombinant IFN-α has been the key component of treatment for chronic HCV infection. Pegylation of IFN-α and its use in combination with RBV has markedly improved treatment efficacy, when compared with standard IFN. Combination therapy with PEG-INF and RBV has long been the standard of care for chronic HCV infection, given for either 48 - 72 weeks (genotypes 1, 4) or 24 - 72 weeks (genotypes 2, 3, 5 and 6) (66). No recommendations have been suggested for genotype 7. However, the treatment is expensive and is associated with significant adverse effects, some of which may be life threatening. The mechanism of action, guidelines for use, response rates, adverse effects and the factors affecting response to therapy has been discussed in detail in a later section.

2) Direct- acting antivirals:

These new antiviral drugs have lately become established components of treatment regimens for chronic HCV infection, especially with genotype 1. Though yet to be introduced in most developing nations, these drugs are revolutionizing the treatment of hepatitis C in the developed world. With their better response rates and lesser side effects, they might replace IFNs in chronic hepatitis C treatment in the next few years (67). The first drugs of this class to be approved were telaprevir and boceprevir, both being NS3/4A protease inhibitors. A combination of these drugs with PEG-INF and RBV for previously untreated genotype 1 infections showed SVR rates of upto 75%. However these drugs are not of great help in cases which have failed previous IFN based treatment. Their spectrum of action is limited to genotype 1 and they need to be combined with the conventional standard of care therapy. These factors led to the introduction of two

newer direct-acting antiviral agents, sofosbuvir, and simeprevir. Sofosbuvir inhibits NS5B RNA-dependent RNA polymerase, and is thus active against all HCV genotypes. Clinical trials have found response rates in various HCV genotypes to vary from 50% to >90%. Simeprevir, a second generation NS3/4A protease inhibitor, has shown response rates of about 80% in previously untreated as well as treatment failed genotype 1 infections (68).

7. Interferon and Ribavirin for treatment of chronic hepatitis C

1) IFN-α monotherapy:

Even before the identification of HCV, IFN-α was shown to benefit patients with *non-A non-B hepatitis* in 1986 (69). Once the virus was identified and diagnostic tests for it were developed, the mechanism of action of IFN and its basis of use was elucidated. IFN-α therapy led to a rapid fall in serum viral load, and resolution of the infection. For the entire following decade, monotherapy with IFN-α was accepted as the standard of care for chronic infection with HCV. However, the sustained response rates were limited to 10 to 25% even after modifications of the dosing regimens and duration of treatment (70). Another issue was severe side effects associated; asthenia, neutropenia, myalgia, headache, thrombocytopenia, and depression (71).

2) IFN-α and Ribavirin combination:

It was by the end of 1990s when RBV, a nucleoside analogue with a broad-spectrum antiviral activity, was introduced for the treatment of chronic HCV infection as a combination with IFNα. This combination therapy showed not only doubled virologic response rates (35–40%), but also improved biochemical and histologic response.

(72). In 1998, a large randomized controlled trial on 912 chronic hepatitis C patients showed a greater than 20% increase in virological response rate with RBV combination therapy over IFNα monotherapy (73). Thereafter this combination therapy became the new standard of care.

3) Pegylated IFN-α:

Further improvement in response rates was brought about in 2001 by the introduction of PEG-IFN-α in combination with RBV. The covalent attachment of a polyethylene glycol moiety to recombinant IFN-α improves the half-life, pharmacokinetic profile and virological response rates (74). This led to the change of the dosing regimen from thrice weekly to the more convenient once-a-week injection. Using the PEG-INFα and RBV combination, sustained virological response (SVR) rates of 40–50% are seen with genotype 1, and ≥80% in genotypes 2 and 3 (8).

7.1 Mechanism of action

7.1.1 Interferon

Interferons are classified as type I, II and III. IFN-α/β/ω are classified as type I, IFN-γ as type II and IFN-λ as type III interferons. All type I IFNs possess antiviral and immunomodulatory activities, but with varying potencies (75). Current therapy for chronic HCV infection banks on the antiviral activity of IFN- α. As has been discussed in the section on innate antiviral response, IFN-α acts by induction of ISGs through intracellular cascades, which create antiviral state within the cell. It does not inhibit viral replication directly. Apart from the induction of ISGs, it also has immunomodulatory effects like activation of NK cells, maturation of dendritic cells, induction of cytokine production, prevention of T cell apoptosis and improved antigen presentation. It is assumed that exogenously supplied recombinant IFN-α works by the same mechanism as endogenous IFN, but with a better effectiveness owing to the higher concentration

supplied. The mechanisms by which the virus can evade the action of interferon have been discussed in detail in a previous section, and include inhibition of the transcription of interferon induced antiviral genes by the HCV core protein and inhibition of the interferon amplication loop by HCV NS3/4A protease (9).

In contrast to type I IFN which are secreted by all virus infected cells, IFN-γ (Type II) is produced by cytotoxic T cells and NK cells and exerts its antiviral action by independent pathways leading to inhibition of viral protein synthesis and RNA replication (76).

Type III interferons (IFN-λ 1, 2 and 3) share great functional similarity with type I IFNs, but have more restricted tissue specificity. Although they engage a distinct receptor, the downsteam signalling pathway is the same (77).

7.1.2 Ribavirin

After its synthesis in 1970, the first approved use of RBV was for the treatment of respiratory syncytial virus infection. On account of its broad spectrum antiviral activity, it was tried as monotherapy, and then as a combination therapy with IFN for chronic hepatitis C, showing sizeable improvement in response rates. The exact mechanism by which RBV acts is not yet known, but a number of theories enjoy experimental support. Some of the accepted mechanisms are listed below (9).

1) Being a guanosine analogue, it gets phosphorylated intracellularly, and is then misincorporated into nascent viral RNA resulting in premature chain termination and inhibition of replication.

2) Ribavirin monophosphate competitively inhibits inosine monophosphate dehydrogenase, leading to depletion of the GTP essential for viral RNA synthesis.

3) RBV reduces the replication efficiency of the virus, thus acting as a viral mutagen and leading to reduced virion infectivity.

4) It is believed to modulate the TH_1/ TH_2 balance towards the TH_1 type response, which is associated with viral clearance.

7.2 Virological response and viral kinetics

Serial monitoring of viral load in patients on therapy is done to assess and prognosticate the treatment response. There are specified time points during the course of treatment at which viral load should be measured. The response definitions and treatment milestones are discussed below (66).

Table 2.Response definitions and treatment milestones

Treatment response or milestone	Definition
Rapid virologic response (RVR)	No detectable HCV RNA in plasma at treatment wk 4
Early virologic response (EVR)	≥2 $\log_{10}$ fall in HCV RNA in plasma at treatment wk 12
Extended rapid virological response (eRVR)	No detectable HCV RNA at 4 wk (rapid) and 12 wk (extended) of treatment
Delayed virological response (DVR)	≥2 $\log_{10}$ fall but detectable HCV RNA at treatment wk 12 and an undetectable HCV RNA at wk 24
End of treatment response (ETR)	No detectable HCV RNA in plasma at end of treatment (depending upon genotype and response)
Sustained virologic response (SVR)	No detectable HCV RNA in plasma at six months after end of treatment

Virological responses to IFN-α based treatments are divided into three broad groups (67).

1) On-treatment response with SVR after treatment

No detectable HCV RNA in plasma at six months after end of treatment

2) On-treatment response and relapse

Undetectable levels of HCV RNA in the plasma of the patient while on treatment but detectable HCV RNA after the treatment is stopped

3) Non-response

(a) Null response:

Less than 2 $\log_{10}$ fall in HCV RNA levels in the plasma of the patient at 12 weeks of treatment.

These patients are considered to be true non‑responders to PEG-INFα and RBV therapy.

(b) Partial response:

Greater than 2 $\log_{10}$ fall in HCV RNA levels at 12 weeks of treatment, but HCV RNA remains detectable throughout the entire course of treatment.

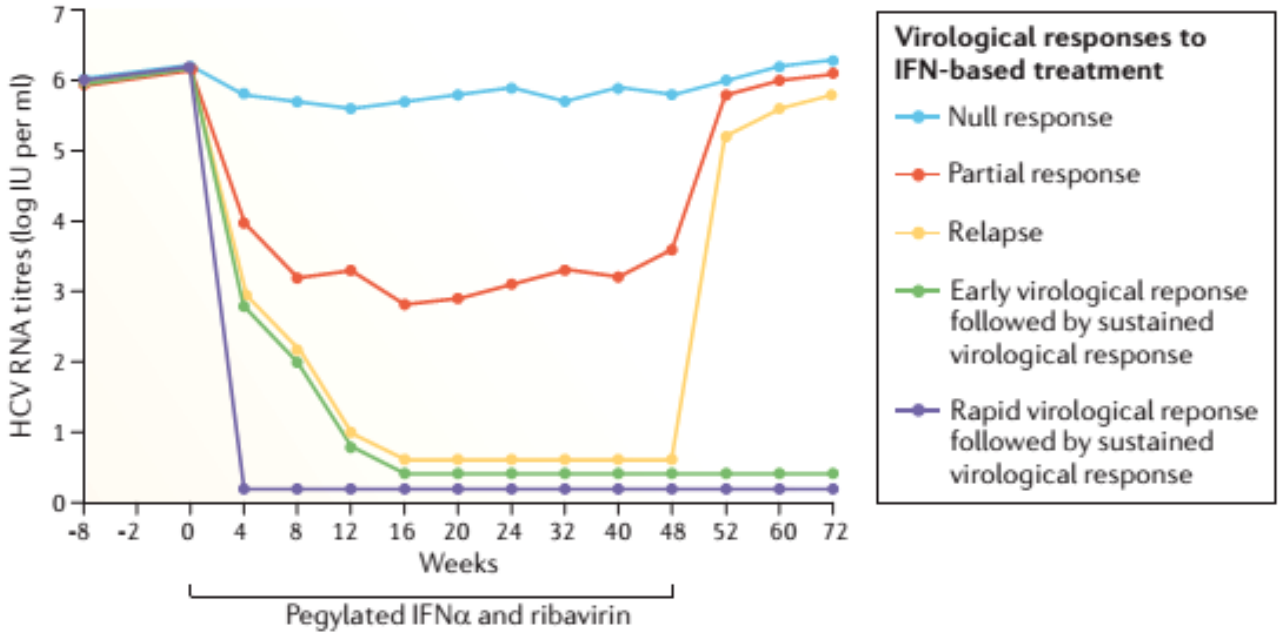


Figure 6.Virological responses following IFN-α based treatments for chronic hepatitis C, Adapted from Heim, (67)

These different response patterns have different implications. An SVR has been found to be associated with a long term response and viral clearance in more than 95% of cases in several studies where the cases were followed up for 5 to 13 years (78,79). Additionally, marked histologic improvement has been seen following viral clearance. A transient response with relapse is seen in less than a quarter of patients on treatment. Retreatment may sometimes benefit such patients, but mostly needs a longer course or higher doses (80). Lastly, about a third of the patients show non-response to treatment. HCV RNA remains detectable throughout the course of the treatment and thereafter, though titres may show some decline. A number of factors have been related to non response and relapse, and have been discussed in detail in a separate section.

7.3 Treatment guidelines for PEG-INF and RBV combination therapy

The duration of treatment required is dependent on the genotype and the response seen. WHO has released guidelines for care and treatment of chronic hepatitis C patients in April 2014, as discussed below (66).

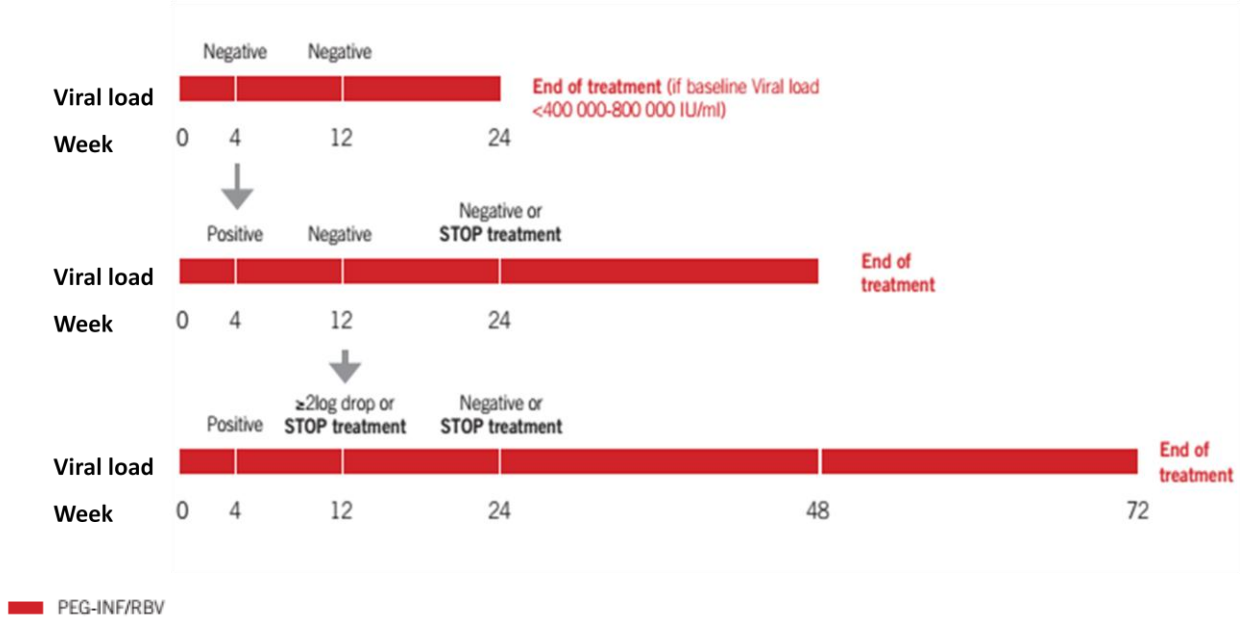


Figure 7.Duration of PEG-INF and RBV therapy for infection with HCV genotypes 1 and 4

The treatment duration with PEG-INF and RBV combination may be varied depending on the response to treatment. If RVR is achieved and pre-treatment viral load is less than 400,000 IU/mL, treatment duration can be reduced to 24 weeks. If viral load is detectable at 24 weeks of therapy, stopping the treatment is recommended. On the contrary, if the patient is showing a slow response with a ≥2 log drop at 12 weeks of treatment and DVR at week 24, a prolonged treatment for 72 weeks can be considered.

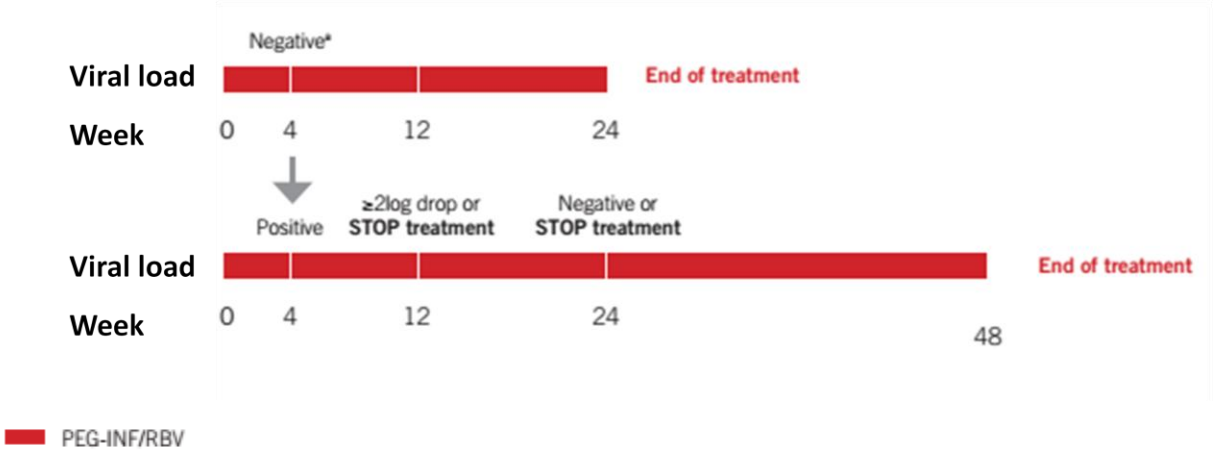


Figure 8.Duration of PEG-INF and RBV therapy for infection with HCV genotypes 2, 3, 5 and 6

RVR is associated with a high probability of SVR, so a short treatment for 24 weeks is sufficient. If the viral load shows less than 2 log drop, or is positive at 24 weeks of therapy stopping the treatment is recommended. On the contrary, if the patient is showing a slow response with a ≥2 log drop at 12 weeks of treatment and DVR at week 24, a prolonged treatment for 48 weeks can be considered.

7.4 Adverse effects of PEG-INF and RBV

A major factor limiting therapy with PEG-INF and RBV is the severe adverse effects associated with it, which often leads to premature withdrawal from treatment. Most patients experience flu-like symptoms soon after the first dose, but that settles in a couple of weeks. Interferon-α commonly causes transient bone marrow suppression, leading to neutropenia, thrombocytopenia and anaemia. These haematological abnormalities may warrant dose reduction or administration of blood cell growth factors (81). The most difficult to manage are the neuropsychiatric side effects such as acute psychosis, anxiety, memory loss, depression, sleep disturbance and

cognitive changes. A combination of counseling, antidepressants and anxiolytic agents may be needed (82). Other less common adverse effects include alopecia, severe skin rash, hyper or hypothyroidism, disordered glucose metabolism, interstitial pneumonitis and ophthalmological abnormalities. Marked interaction with other drugs may be seen. RBV causes dose dependant haemolytic anaemia and is a known teratogen. The use of IFN and RBV is contraindicated in a number of conditions like transplant recipients, autoimmune hepatitis, active psychiatric illness and untreated hyperthyroidism and severe uncontrolled concurrent diseases like hypertension, diabetes, epilepsy, chronic obstructive pulmonary disease, coronary artery disease, haemoglobinopathies etc.

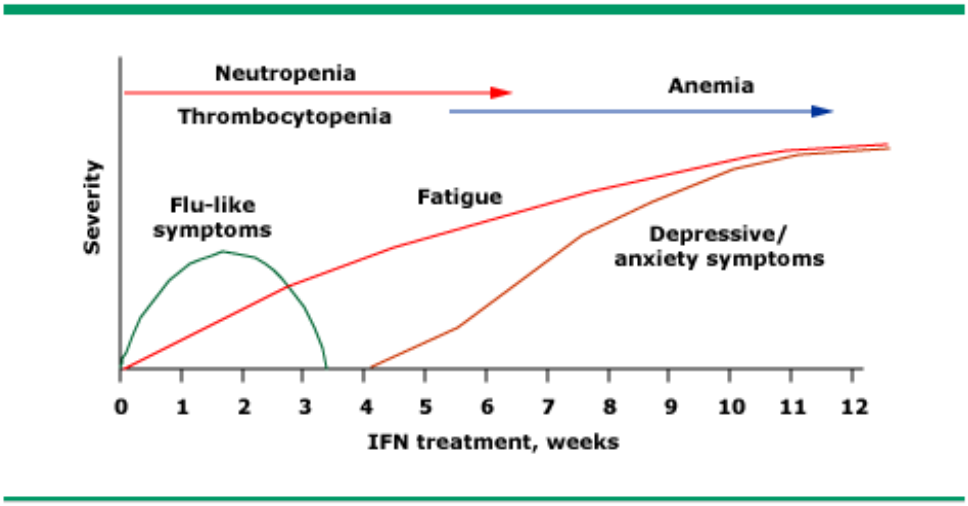


Figure 9.The time course of side effects associated with interferon treatment

8. Factors affecting response to treatment

The goal of therapy in chronic HCV infection is the attainment of SVR, which predicts eradication of HCV RNA and decreased complications. A number of factors are known to influence the response to interferon based therapies and, therefore the likelihood of an SVR. These factors are broadly classified as viral and host factors, and are discussed in detail below.

8.1 Viral factors

1) Viral genotype

The most important viral factor that has a bearing on response to interferon based therapy is the genotype of the infecting virus. Though the underlying functional mechanism is unknown, there is an inherent difference in response to treatment among the various genotypes of HCV, which is the reason for the different treatment durations needed for them. Many large trials have attempted to study and compare the response rates in the various genotypes. The SVR rates for PEG-INF and RBV combination therapy have been estimated as follows.

Table 3.Rates of SVR in different genotypes of HCV

HCV Genotype	SVR rates (PEG-INF + RBV)	References
Genotype 1	41-52%	(83–87)
Genotype 2 and 3	65-80%	(85,87–89)
Genotype 4	50-70%	(90,91)
Genotype 5	63-67%	(92–94)
Genotype 6	62-80%	(95,96)

2) Baseline viral load

Pre-treatment viral load has been found to be an independent predictor of treatment response for all genotypes. Lower baseline viral loads (≤600,000 to 800,000 IU/mL) are associated with greater response rates (83,86,88,97).

3) Viral quasispecies

An increased degree of quasispecies heterogeneity is associated with a lower probability of SVR (9). It has been found that during treatment quasispecies decrease rapidly in the patients who attain SVR (98).

8.2 Host factors

1) Age

It has been shown in large multicenter clinical trials that younger patients show better response rates to treatment. Fried *et al.*(87) in their randomised clinical trial involving 1121 patients of genotypes 1 to 6 found age ≤ 40 years to be significantly associated with the achievement of SVR (odds ratio 2.60; 95 percent confidence interval 1.72 to 3.95 and P<0.001). In another large study by Shiffman *et al.*(89) on 1465 genotype 2 and 3 patients, age ≤ 45 years was predictive of SVR (odds ratio 1.50; 95% confidence interval 1.17 to 1.93 and P = 0.002). The poorer response in the older patients is believed to be attributable to the more extensive liver damage owing to the longer duration of the disease in them (83).

2) Gender

Female gender has been linked to a better response to treatment (9,67) while some studies have not been able to establish a significant association (87,89).

3) Ethnicity

Ethnicity also has a significant impact on response to treatment. It has been demonstrated that Asians respond best to interferon based treatment, followed by Caucasians and then African Americans (97). The lowest response rates seen in African Americans was attributed to the commonness of HCV genotype 1 infection in them (83). However, this is being increasingly ascribed to the differential distribution of the IL28B polymorphisms in various ethnic groups (99,14).

4) Body weight and BMI

High body weight is inversely correlated with SVR. Fried *et al.*(87) found that body weight of 75 kg or less was predictive of SVR (odds ratio 1.91; 95 percent confidence interval 1.27 to 2.89; P=0.002). Likewise Shiffman *et al.*(89) found a significant association with weight $\leq$ 80kg (odds ratio 1.75; 95% confidence interval 1.37 to 2.24; P<0.001).

5) Liver fibrosis and steatosis

Advanced liver fibrosis or cirrhosis are major predictors of non-response, across all genotypes (97). In a clinical trial involving 4913 patients by Jacobson *et al.*(85) the odds ratio for SVR in patients with cirrhosis compared to those without cirrhosis was 0.58 (95% CI 0.47-0.73, P <0.0001). Similarly steatosis also impairs the likelihood of achieving SVR (100).

6) Diabetes mellitus

Diabetes mellitus and insulin resistance has emerged as a cofactor in failure to achieve SVR, because of the higher prevalence of steatosis and advanced fibrosis in diabetics(101). Interestingly, successful treatment of HCV infection has shown to reduce the risk of

development of type 2 diabetes by attenuating insulin resistance, restoring pancreatic beta-cell function, and reverting glucose abnormalities in pre-diabetics (102,103).

7) IL28B polymorphisms

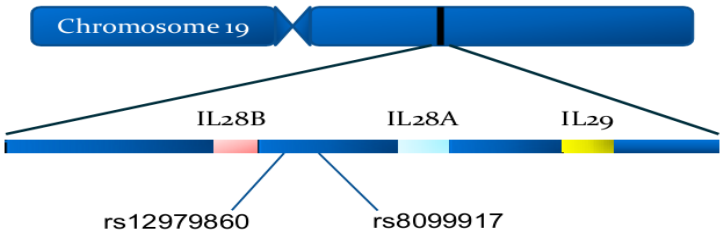
Single nucleotide polymorphisms (SNPs) refer to base-pair variations at a particular genomic location with a minor allele frequency of >1% within a population. Between 2009 and 2010, four independent genome-wide association studies across the world identified SNPs in the vicinity of the IL28B gene on chromosome 19 to be highly predictive of response to PEG-IFN and RBV in chronic HCV infection (10–12,104). These studies included patients of different ethnicities (Caucasian, African American, Australian and Japanese). In the largest of these studies by Ge *et al.*(10) on 1137 patients infected with HCV genotype 1, several IL28B polymorphisms encoding IFN-λ3 were identified to be significantly more common in responders than in non responders, in patients of both European and African-American ethnicities. It was also suggested that the greater frequency of favourable genotypes in Europeans may be largely responsible for the better response rates seen in them compared to African-Americans.

Biology of IFN- λ

The IL29, IL28A and IL28B genes located on chromosome 19 code for IFN- λ1, IFN- λ2 and IFN- λ3 respectively, which constitute the IFN-λ family which is categorized as type III IFN (IFN-α/β/ω are classified as type I and IFN-γ as type II). The type III IFNs are functionally similar to type I and exert antiviral activity via the same downstream signalling pathway, as discussed in a previous section. However the transmembrane receptors to which these classes of IFNs bind are different which may result in altered kinetics of ISG expression (105).

IL28B SNPs

Of the various SNPs studied, rs12979860 and rs8099917, both in the vicinity of IL28B, have shown the strongest association with treatment response.



rs12979860 is located 3 kb upstream of the IL28B gene, while rs8099917 SNP is located 8 kb downstream. The possible genotypes at rs12979860 are C/C, C/T and T/T, while those at rs8099917 are T/T, T/G and G/G. The C/C genotype of rs12979860 and T/T of rs8099917 have been shown to be associated with a better treatment response (10,104). The exact mechanism by which these polymorphisms impact treatment response is yet to be elucidated, but it has been found that the CC genotype is associated with lower intrahepatic ISG levels, which is linked to better response to IFN based treatment (106). As the two SNPs are in strong linkage disequilibrium, rs8099917 is believed to act by similar mechanisms (107).

IL28B polymorphisms and spontaneous clearance of HCV infection

Spontaneous clearance of HCV is seen in a very small number of cases, while chronicity is the major outcome. Thomas *et al.* (14) determined IL28B rs12979860 polymorphisms in large HCV cohorts, consisting of patients who had cleared the infection spontaneously (n = 388) and those who had persistent infection (n = 620). They found that the C/C genotype was associated with about three fold higher clearance rates than the non C/C in patients of both European and African

ancestry. Similar were the findings in another homogeneous German HCV infected cohort (108). In their genome wide association study, Rauch *et al.* found rs8099917 polymorphisms to impact spontaneous clearance not only in HCV mono-infected but also in HCV/HIV coinfected individuals (104).

IL28B polymorphisms and treatment response in HCV genotype 1 and 4 infections

After the genome wide association studies established the significance of these polymorphisms in genotype 1 infection, several studies replicated the association (109,110). Thompson *et al.* in their study on 1587 HCV genotype 1 infected patients established rs12979860 as the strongest pre treatment predictor of SVR (111). Significant association of the polymorphism with treatment response has also been found in genotype 4 infections subsequently (15,112). A meta-analysis of 11 studies encompassing 1284 HCV 4 mono-infected patients found rs12979860 genotype CC and rs8099917 genotype TT to be statistically significant predictors of SVR (113). Jia *et al.* in their meta-analysis of 34 papers have found these SNPs to be associated with response in genotypes 1 and 4, but not for 2 and 3 (107). A larger meta-analysis of 67 studies showed a similar result for genotypes 1 and 4 (16).

IL28B polymorphisms and treatment response in HCV genotype 2 and 3 infections

The association of IL28B polymorphisms with response to treatment in HCV genotype 2 and 3 infections have remained controversial. Mangia *et al.* in their study involving 268 patients (genotype 2= 213; genotype 3= 55) found that the CC genotype was associated with SVR in patients who did not achieve an RVR, but not in those who did (114). In contrast, Sarrazin *et al.* studied 267 patients (genotype 2= 77, genotype 3= 190), and found an association between IL28B CC type and SVR in the subgroup of patients who achieved RVR. No association was

observed in patients who did not attain RVR (115). In two studies on genotype 3 patients exclusively, both rs12979860 and rs8099917 could not be associated with SVR (116,117). Similarly, the results of meta-analyses in this group have also been conflicting. Jia *et al.* (107) in their meta-analysis concluded that these polymorphisms did not have an association with PEG-INF and RBV therapy in genotypes 2 and 3. Chen *et al.* found no association of rs12979860 with SVR, but showed that TT at rs8099917 SNP was predictive of a favourable response in genotype 2 infected Asian patients (118). Another meta-analysis found an association with SVR and RVR in Caucasian subjects. On the contrary, the favourable IL28B genotype was found to be predictive of RVR, but not SVR in Asian HCV genotype 2 patients (119). However, the largest meta analysis by Jiménez-Sousa *et al.* found significant associations rs12979860 and rs8099917 polymorphisms with treatment response in genotypes 2 and 3 infected patients, but the strength of association was three fold lower than that for genotypes 1 and 4 (16). It has been suggested that the inherently higher treatment response rates in these genotypes might attenuate the effect of SNPs, and this necessitates the study of larger sample sizes to find significant differences (16,120).

The Indian scenario

In 2012, Sivaprasad *et al.* (17) studied the distribution of genotype and allelic frequency of IL28B rs12979860 polymorphism in 220 healthy uninfected controls in Andhra Pradesh, India, and found that the frequency of CC genotype (59%) was significantly higher compared to CT (34.09%) and TT (6.81%). However the association of these SNPs with treatment response was not looked into. Thereafter two studies have attempted to find out the association of this SNP in HCV genotype 3 patients.

Gupta *et al.* (18) from New Delhi analysed the rs12979860 SNP in 356 patients infected with HCV genotype 3 and found the CC genotype to be an independent strong predictor of RVR and SVR. The rs12979860 genotype CC/ CT/TT distribution was 58.3%, 34.5%, and 7.2% respectively. SVR was seen in 250 (70.2%) patients. The non-CC genotypes were found in 67.6% in non-responders, compared to a much lower 38.9% in responders ($P<0.001$). 360 matched uninfected individuals served as healthy controls, in whom the genotype CC/CT/TT distribution was found 60.6%, 35.2%, and 4.2% respectively.

In a study by Firdaus *et al.* (19), 400 HCV seropositive patients from Eastern and North Eastern India were genotyped for rs12979860 and rs 8099917 polymorphisms. Out of the total, 293 (73.25%) were positive for HCV RNA (genotype 3=193, genotype 1=94 and genotype 6=6). IL28 B genotyping was done for all 400 cases. The frequency of CC/ CT/TT genotype at rs12979860 was 70.75%, 24% and 5.25%, while that of TT/TG/GG genotype at rs8099917 was 77.50%, 15% and 7.50 respectively. In the control group including 100 healthy individuals, the distribution at locus rs12979860 was 73%, 23% and 4% for CC/ CT/TT. At rs8099917 the favourable genotype TT was found in 70% individuals, followed by TG in 30%. There were no GG alleles in this group.

Among the 293 viral RNA positive individuals, 83 genotype 3 infected individuals were treated with PEG-INF and RBV. SVR was achieved in 46 out of 83 individuals (55.42%) while RVR in 26 individuals (31.3%). Genotypes CC at rs 12979860 and TT at rs8099917 were found to be strongly associated with SVR.

This study aims to determine the frequency and distribution of IL28B gene polymorphisms in patients with chronic HCV infection harbouring genotype 1 in addition to 3, and to study the association of these SNPs with response to IFN based treatment.

MATERIALS AND METHODS

Study Design

This is an observational study conducted in the Department of Clinical Virology, Christian Medical College, Vellore. Patients infected with HCV on treatment with Interferon (standard / pegylated) and RBV were recruited from the Liver Clinic and in-patient wards of the Department of Hepatology.

Ethics approval:

The approval for the study was obtained from the Institutional Review Board, CMC, Vellore (IRB Min No: 8202 dated 13.02.13).

Study duration:

The study was conducted over a period of 17 months from April 2013 to August 2014.

Study samples:

The study subjects comprised of individuals attending the Liver Clinic of Christian Medical College, Vellore, on treatment for HCV infection. These subjects were referred to the Department of Clinical Virology for routine hepatitis C viral load testing and /or HCV genotyping. Written informed consent was obtained from the patient at the time of blood collection for routine testing.

Inclusion Criteria:

1. ≥18 years of age
2. Patients with HCV genotype 1, 3 and 4 infection

3. On therapy with Interferon (standard or pegylated) and RBV

Exclusion Criteria:

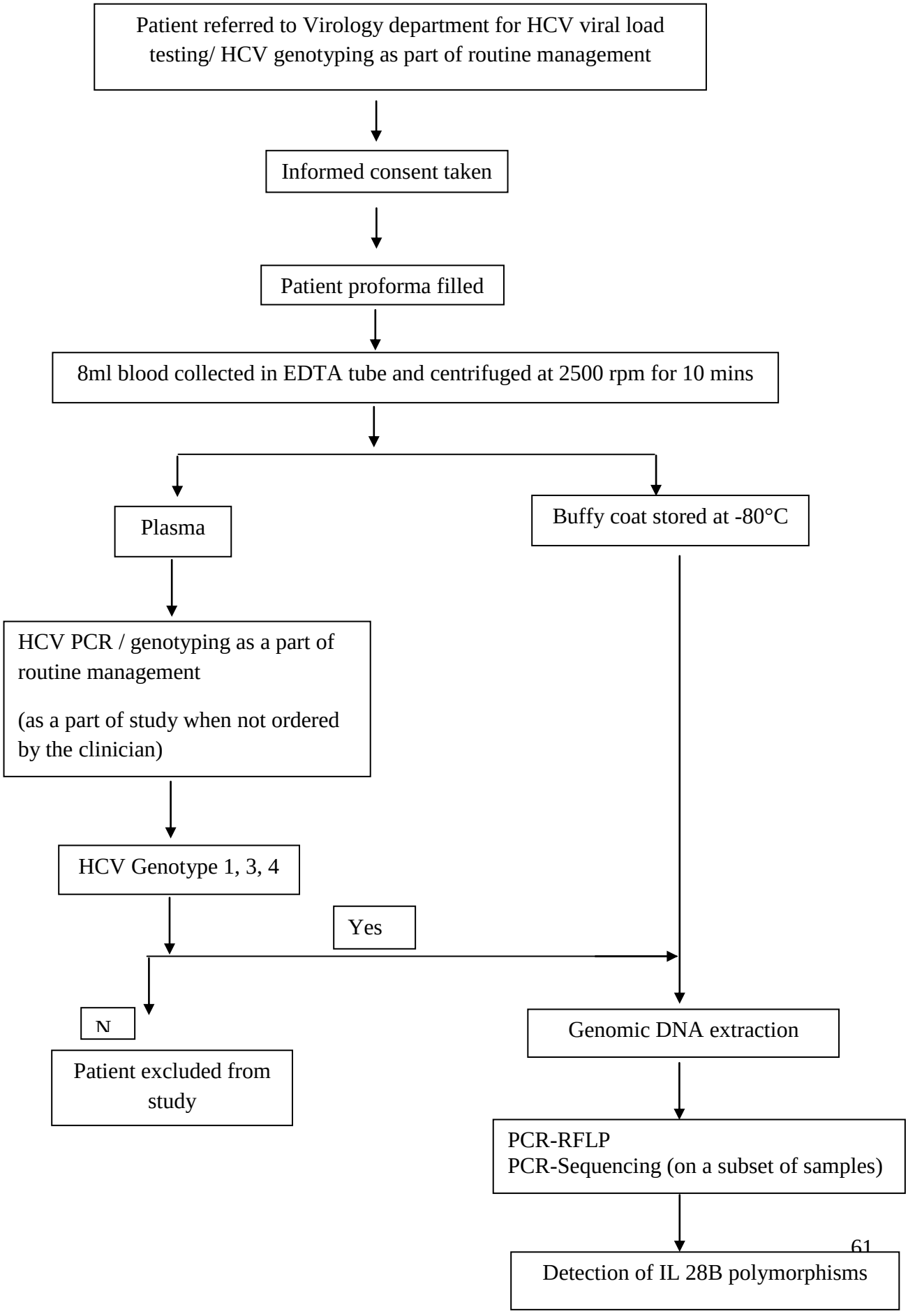
1. Immunosuppressed patients
2. Hepatitis B or HIV co-infection
3. Patients on dialysis
4. Pregnancy
5. Tuberculosis

Data sources:

Relevant information about the patient, like age, sex, address, weight, height, diabetic status, ALT levels, was obtained from clinical records. Testing of the study samples was done in the Department of Clinical Virology.

Study algorithm

Diagrammatic representation of the study algorithm is shown below.


Patient referred to Virology department for HCV viral load testing/ HCV genotyping as part of routine management
Informed consent taken
Patient proforma filled
8ml blood collected in EDTA tube and centrifuged at 2500 rpm for 10 mins
Plasma
Buffy coat stored at -80°C
HCV PCR / genotyping as a part of routine management
(as a part of study when not ordered by the clinician)
HCV Genotype 1, 3, 4
Yes
N
Patient excluded from study
Genomic DNA extraction
PCR-RFLP
PCR-Sequencing (on a subset of samples)
Detection of IL 28B polymorphisms

1. Processing of study samples

8 to 10 mL of blood was collected by venipuncture in vacutainer tubes containing dipotassium ethylene diamine tetra acetate (K_2EDTA) (BD Biosciences). The tube was centrifuged at 2500 rpm for 10 minutes and the supernatant plasma was transferred out with a pipette. This was stored at -60°C HCV viral load testing and/or genotyping as a part of routine management. Then the buffy coat layer was carefully pipetted out was and was added to an aliquot of cell freezing solution to make up a volume of 200 µl. This was stored at -60°C until testing.

Preparation of the cell freezing solution

The glycerol cell freezing solution was prepared as per the guidelines by Austin *et al*. (121)

Requirements

Reagent	**Amount per 100 ml**
Citric acid, sodium salt	1.47 gm
Sodium phosphate, monobasic monohydrate	0.276 gm
Sodium phosphate, dibasic anhydrous	0.284 gm
99% glycerol	40.00 ml

1. The salts were weighed and added to 40 ml glycerol.

2. The volume of the solution was adjusted to slightly less than 100 ml using sterile distilled water.

3. Concentrated sodium hydroxide was added to bring the pH to 7.4.

4. The final volume was brought to 100 ml using sterile distilled water.

5. The solution was filtered, aliquoted into micro centrifuge tubes, 1ml each, and stored at 4°C.

2. DNA extraction from the buffy coat

A. Materials required

Buffy coat saved in cell freezing solution (200 µl)

QIAamp® DNA Blood Mini Kit (Qiagen, Hilden, Germany) containing:

- Proteinase K
- Lysis buffer AL
- Wash buffer 1 AW1
- Wash buffer 2 AW2
- Elution buffer AE
- QIAamp Mini Spin Columns
- Collection Tubes (2 ml)

Ethanol

Nuclease free water

Dry bath (temperature to be set at 56°C)

B. Procedure

DNA extraction was performed in the 'PCR dirty' room as per manufacturer's instructions.

1. The samples were equilibrated to room temperature and dry bath was set to heat to 56°C.

2. Proteinase stock solution was prepared by adding 1.2 ml of proteinase solvent into the vial containing lyophilized protease. The solution was stored at 2-8°C.

3. 20 µl of Proteinase K was pipetted into a 1.7 ml micro centrifuge tube, to which 200 µl of the thawed sample was added.

4. 200 µl of AL was added to the tube and mixed well by pulse vortexing for 15 seconds.

5. The tube containing the above mixture was incubated in a dry bath at 56°C for 10 minutes.

6. The tube was then centrifuged briefly to remove moisture from the inside of the lid.

7. 200µl of 100% ethanol was added to the sample and mixed well by pulse-vortexing for 15 second and briefly spun down.

8. The entire content (620 µl) was transferred to the spin column (silica-gel membrane) and centrifuged at 8000rpm for one minute.

9. The filtrate along with the collection tube was discarded and the mini spin column was placed on a fresh collection tube.

10. 500µl of AW1was added to the mini spin column and centrifuged at 8000rpm for one minute.

11. The collection tube with the filtrate was discarded and the mini spin column was placed on a fresh collection tube.

12. 500µl of AW 2 was added to the spin column and this was centrifuged at 14,000 rpm for 3 minutes.

13. The collection tube with the filtrate was discarded and the mini spin column was placed on a fresh collection tube and centrifuged at 14,000 rpm for 1 minute.

14. The collection tube was discarded and the spin column was placed in a 1.5 ml micro centrifuge tube. 100µl of elution buffer AE was added to the spin column and incubated at room temperature for one minute, and then centrifuged at 8000rpm for one minute.

15. The DNA extract was stored in aliquots at -20°C.

3. Genomic DNA quantification

The extracted DNA was quantified spectrophotometrically using Take3, Gen5^{TM}, Biotek. 2 µl of the extracted DNA was loaded on the Take3 microplate and readings were taken using the Gen5^{TM} software. Both the concentration and purity of DNA was determined.

4. IL28B genotyping by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

Principle:

Polymerase chain reaction amplifies a specific target region of the template DNA strand. RFLP is based on the digestion of the PCR product by restriction endonucleases at specific sites depending upon nucleotide variations at the site of polymorphism. This produces DNA fragments of precise lengths giving discrete electrophoretic patterns based upon their sizes. Using suitable primers and cycling conditions the two target sites rs12979860 and rs8099917 were amplified separately. Digestion of the PCR products with restriction enzymes was carried out and the two polymorphisms were detected depending upon the electrophoretic patterns produced.

4.1. PCR

4.1.1. Primers

Primers for the amplification of rs12979860 and rs8099917 were the same as that used by Sharafi *et al*.(122)

rs12979860

Forward primer	5’ GCGGAAGGAGCAGTTGCGCT 3’
Reverse primer	5’ GGGGCTTTGCTGGGGGAGTG 3’

rs8099917

Forward primer	5’ CCCACTTCTGGAACAAATCGTCCC 3’
Reverse primer	5’ TCTCCTCCCCAAGTCAGGCAACC 3’

The lyophilised primers were reconstituted using Tris EDTA (TE) buffer.

4.1.2. Master mix

The concentrations of the different components of the PCR mix for one reaction are given below:

Reagents	Volume per reaction (µl)
HotStar Taq master mix (Qiagen, Hilden, Germany)	12.5
Forward primer	1 (10 pmol/ µl)
Reverse primer	1 (10 pmol/ µl)
Nuclease free water	5.5 to 9.5
DNA extract (100 to 300 ng)	1 to 5
Total volume	25

4.1.3. Procedure for amplification

1. The master mix was prepared for the appropriate number of reactions with the above template. The master mix was prepared in the clean room or 'DNA-free' room. Separate master mixes were prepared for the two reactions.

2. Appropriate number of 0.5ml PCR tubes were labeled appropriately, and the master mix was distributed in them.

3. DNA extracts were removed from the storage area, brought to room temperature and spun briefly in a microcentrifuge.

4. 1 to 5 µl of DNA was added, depending upon the concentration. The final volume was 25 µl. Addition of DNA was done in the 'dirty room'.

5. Nuclease free water was used as negative control after every three samples.

6. Amplification reactions were carried out in Veriti ™ Thermal Cycler (Applied Biosystem, Foster City, California, USA)

7. The cycling conditions were:

rs12979860

Initial denaturation: 95°C for 15 minutes

Denaturation: 95°C, 30 sec
Annealing: 60°C, 30 sec
Extension: 72°C for 45 min
} X 40 Cycles

Final extension: 72°C for 7 min

rs8099917

Initial denaturation: 95°C for 15 minutes

Denaturation: 95°C, 30 sec
Annealing: 56°C, 30 sec
Extension: 72°C for 45 min
} X 40 Cycles

Final extension: 72°C for 7 min

8. The length of the expected amplification products were

rs12979860: 241 base pairs rs8099917: 552 base pairs

4.1.5. Post amplification DNA detection by Gel Electrophoresis:

1. Five microlitres of each amplicon was mixed with 1 µL of 6X loading dye bromophenol blue.
2. The amplified products were then subjected to electrophoresis in freshly prepared 2% agarose gel containing 0.5 µg/ml ethidium bromide.
3. Test samples and negative control were loaded in appropriate wells. Molecular ladder (DNA Marker-A, Bio Basic Inc.Canada) used was 25-500 bp long.
4. The electrophoresis was done at 120 volts for 35 minutes.
5. The gel was visualized by ultraviolet radiation using Quantity one® (version 4.6.2) software in the gel documentation system (BioRad, Hercules, California, USA).

4.2. Restriction Fragment Length Polymorphism

For RFLP analysis, digestion of the amplicons was carried out using restriction endonucleases BstUI (*New England Biolabs*, UK) for rs12979860 and BsrDI (*New England Biolabs*, UK) for rs8099917.

The reaction volumes were as follows.

rs12979860 analysis		**rs8099917 analysis**	
Reagent	Volume per reaction (µl)	Reagent	Volume per reaction (µl)
BstUI	2	BsrDI	2
Buffer	2	Buffer	2
Nuclease free water	8	Nuclease free water	8
Amplified product	8	Amplified product	8
Total volume	20	Total volume	20

The reactions for rs12979860 were incubated at 60°C for 15 minutes, and those for rs8099917 at 65°C for mins Veriti ^{TM} Thermal Cycler (Applied Biosystem, Foster City, California, USA).

Gel Documentation following RFLP

Ten microlitres of each amplicon was mixed with 2 µL of 6X loading dye bromophenol blue and then subjected to electrophoresis in freshly prepared 3% agarose gel containing 0.5 µg/ml ethidium bromide. Molecular ladder (DNA Marker-A, Bio Basic Inc.Canada) used was 25-500 bp long. The gel was visualized by ultraviolet radiation using Quantity one® (version 4.6.2) software in the gel documentation system (BioRad, Hercules, California, USA).

Interpretation of electrophoretic patterns after RFLP

rs12979860

Bands obtained	Genotype
196, 45 base pairs	CC
241, 196, 45 base pairs	CT
241 base pairs	TT

rs8099917

Bands obtained	Genotype
552 base pairs	TT
552, 322,230 base pairs	GT
322, 230 base pairs	GG

5. IL28B genotyping by polymerase chain reaction-sequencing

A representative set of samples (5 each for rs12979860 and rs8099917) were bidirectionally sequenced by Sanger sequencing for verification of RFLP results.

Principle:

This is a 'chain termination' method of DNA sequencing to determine the precise order of nucleotides in a segment of DNA. After amplification of the target region, the products are purified in order to remove extra dNTPs and primers (Pre-cycle sequencing clean-up). This is followed by PCR to synthesize single stranded DNA templates. The reaction mix for the PCR contains, along with the four deoxynucleotide triphosphate (dNTPs), limited amounts of different dideoxy nucleoside triphosphate (ddNTPs). These ddNTPs get incorporated into the chain by DNA polymerase, as efficiently as dNTPs. However, as they lack the 3'-OH group required for attachment of the next nucleotide, their incorporation brings about chain termination. This process generates fragments randomly that differ in length by one base pair. In automated cycle sequencing using dye terminator chemistry, the ddNTPs are tagged with different fluorescent dyes, each emitting light at a unique wavelength when excited by a laser. As the products are subjected to capillary electrophoresis in a genetic analyzer, a laser excites the fluorescent dye labels at the 3' ends of the ddNTPs, and depending upon the unique wavelength emitted, the nucleotide is identified as adenine (A), cytosine (C), guanine (G) or thymine (T). The emitted fluorescence is recorded by a camera and a software converts the data to a colour coded electropherogram, in which blue represents C, green represents A, black represents G and red represents T.

5.1. DNA amplification by PCR

1. The primers used were the same as those for PCR-RFLP, except the reverse primer for rs12979860, which was as follows: 5' GTGCCTTCACGCTCCGAGCA 3'

2. The master mix preparation was done in the same manner as PCR-RFLP. The cycling conditions were also the same.

3. Amplification of rs12979860 produced a 746 base pair product while rs8099917 amplification produced a 552 base pair product.

4. Electrophoresis of the amplified products was done on 2% agarose gel.

5. The gel was visualized by ultraviolet radiation using Quantity one® (version 4.6.2) software in the gel documentation system (BioRad, Hercules, California, USA).

5.2. Pre-cycle sequencing clean-up

1. After gel documentation to ensure optimal amplification, 80 µl of nuclease free water was added to the amplicon to make the volume 100 µl.

2. The diluted product was transferred to the Multiscreen HTS PCR plate (Millipore, Billerica, USA).

3. The pre-sequencing plate was placed on the Millipore vacuum manifold and vacuum pressure was applied until the well was completely dry.

4. The above step was repeated with 100 µl of nuclease free water.

5. After the wells dried completely, 10 µl of nuclease free water was added and mixed in titer plate shaker (Barnstead International, Dubuque, USA) for 2 minutes at 7000 rpm.

6. Thus 10 µl of purified DNA was eluted, and transferred to PCR tubes.

5.3. Sequencing PCR

1. Sequencing reactions were carried out for the forward and reverse strands using ABI Prism BigDye® terminator v3.1 cycle sequencing reagents.

2. The reaction volumes were as follows.

Reagent	Volume per reaction (µl)
Ready reaction (RR) mix	0.5
Sequencing buffer	1.75
Primer (Forward/Reverse)	2 (1 pmol/µl)
Purified PCR product	1
Nuclease free water	4.75
Total volume	10

3. The cycling conditions were

96°C for 15 seconds
50°C for 20 seconds
60°C for 4 minutes
} 25 cycles

5.4. Post cycle sequencing clean-up

1. 30 µl of injection solution was added to the sequence reaction samples.

2. The diluted reactions were transferred onto Montage SEQ96 filtration plates (Millipore Billerica, USA).

3. The plate was placed on the Millipore vacuum manifold and vacuum was applied until the wells were completely dry.

4. 40 µl of injection solution was added to the wells and the step was repeated.

5. After the wells dried completely 30 µl of injection solution was added and mixed in titer plate shaker (Barnstead International, Dubuque, USA) for 2 minutes.

6. 30 µl of the resuspended DNA thus obtained was transferred to genetic analyzer sample tubes and sealed with septa.

5.5. DNA sequencing and sequence analysis

ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, California, USA) was used for the DNA sequencing. Bidirectional sequences were obtained for both the targets and analyzed using BioEdit sequence alignment editor. Reference sequences for rs12979860 and rs8099917 were downloaded from NCBI SNP database. The obtained sequences were aligned with the reference sequence and the polymorphisms were detected.

6. HCV Genotyping

Genotyping of HCV was done as a part of the study for cases where the test was not ordered as a part of the routine management. This is based upon sequencing of the NS5B region of the HCV genome (123). The procedure involves conversion of RNA into c DNA (reverse transcription), and subsequent amplification of the c DNA. A nested PCR involving two consecutive PCRs is performed.

The steps for the procedure are as follows

6.1. Viral RNA Extraction from plasma

Materials required:

Plasma sample (either freshly separated or stored at -60°C)

QIAamp® Viral RNA Mini kit containing

Buffer AVL

Buffer AW1

Buffer AW2

Buffer AVE

Carrier RNA

Spin columns

Collection tubes (2ml)

100% ethanol

Nuclease free water (to serve as negative control)

Procedure:

The method as recommended by the manufacturers was followed for the extraction process.

1. Before starting the extraction process carrier RNA solution was prepared by adding 310 µl Buffer AVE to the tube containing 310 µg lyophilized carrier RNA. This was thoroughly dissolved, divided into conveniently sized aliquots, and stored at –20°C.

2. For each batch of extraction Buffer AVL and carrier RNA- Buffer AVE mix was freshly prepared, in appropriate proportions (for one sample Buffer AVL = 0.56, carrier RNA- Buffer AVE=5.6).

3. The tubes were labelled appropriately and plasma samples were equilibrated to room temperature.

4. 560 µl of prepared Buffer AVL containing carrier RNA was pipetted into a 1.5 ml microcentrifuge tube.

5. 140 µl of plasma sample was added to the tube and mixed by pulse-vortexing for 15 s.

6. The mixture was incubated at room temperature (15–25°C) for 10 min and then spun briefly to remove moisture from the inside of the lid.

7. 560 µl of ethanol was added to the sample, and pulse-vortexed for proper mixing followed by brief centrifugation.

8. 630 µl of this solution was transferred to the spin column (silica-gel membrane) and centrifuged at 8000rpm for one minute.

9. The filtrate along with the collection tube was discarded and the mini spin column was placed on a fresh collection tube.

10. Remaining 630 µl of the solution in the microcentrifuge was transferred to the spin column, and centrifuged at 8000rpm for one minute. The collection tube with the filtrate was discarded and the mini spin column was placed on a fresh collection tube.

12. 500µl of AW 1 was added to the spin column and this was centrifuged at 8000rpm for one minute, and the filtrate along with the collection was discarded and a fresh tube used.

13. 500µl of AW 2 was then added and centrifuged 14,000 rpm for 3 minutes. The spin column was placed in a clean 1.5 ml microcentrifuge tube and the collection tube containing the filtrate was discarded.

14. 60 µl of Buffer AVE was added to the microcentrifuge tube and after incubation at room temperature for 1 min it was centrifuged at 8000 rpm for 1 min.

15. The spin column was discarded and viral RNA was eluted.

6.2. First round PCR

This was done using Titanium One-Step RT-PCR Kit (Clonetech laboratories, Takara Bio Inc, Japan).

The primer sequences were as follows:

P1203	5’ GGGTTCTCGTATGATACCCGCTGCTTTGACTC 3’
P1204	5’ GGAGGGGCGGAATACCTGGTCATAGCCTCCGTGAA 3’

The master mix was prepared as follows.

Reagent	Volume per reaction (µl)
10X One Step Buffer	5.0
50X dNTP mix	1.0
Recombinant RNAase inhibitor	0.5
Thermostabilizer	25.0
GC melt	10.0
50X Titanium taq polymerase	1.0
Oligo dt primer	1.0
P1203 (50 pmol/ µl)	0.9
P1204 (50 pmol/ µl)	0.9
Total	45.3

Appropriate amount of the master mix was prepared depending upon the number of samples, and was divided into PCR tubes. Nuclease free water was used as a negative control after each sample. 5 µl of the extracted RNA was added to the tube and was taken for amplification using Veriti TM Thermal Cycler (Applied Biosystem, Foster City, California, USA). The cycling conditions used were

50 °C for 60 minutes

94°C for 5 minutes

94°C for 30 seconds
65°C for 30 seconds
68°C for 1 minute
} 35 cycles

68°C for 2 minutes

After amplification, the products were subjected to electrophoresis on a 2% agarose gel.

The amplified products were taken to the second round PCR.

6.3. Second round PCR

Primers used were P1204 (as in first round) and NS5B internal primer.

(5' TGATACCCGCTGCTTTGACTCNACNGTCAC 3')

The master mix was prepared as follows:

Reagent	Volume per reaction (µl)
10 X PCR buffer	5.0
dNTPs (100mM)	0.4
P1204 (50 pmol/ µl)	0.4
NS5B primer (50 pmol/ µl)	0.4
Taq polymerase	0.2
Nuclease free water	38.6
Total	45.0

The master mix was distributed into required number of tubes. 5 µl of the amplified product from the first round PCR was added to the reaction. The samples were amplified using Veriti TM Thermal Cycler (Applied Biosystem, Foster City, California, USA). The cycling conditions used were

94°C for 3 minutes

94°C for 30 seconds
54°C for 40 seconds
72°C for 30 seconds
} 40 cycles

72°C for 3 minutes

Gel documentation for the second round products was done to verify the adequacy of amplification.

6.4 Sequencing and analysis

The second round products were subjected to pre-cycle sequencing clean up, sequencing PCR and post-cycle sequencing clean up, and then loaded onto the auto sampler tray of ABI PRISM 310 genetic analyzer (Applied Biosystems, Foster City, California, USA) (same procedure as described above for IL28 sequencing).

Forward and reverse sequences were obtained from the sequencer, and were aligned together using BioEdit software to get a consensus nucleotide sequence. The obtained sequence was subjected to BLAST using online software HCV BLAST (hcv.lanl.gov). Depending upon the homology found, the genotype of HCV was identified. The sample sequence was then included in a phylogenetic tree that has representative genotypes identified in this centre.

Statistical analysis

Continuous variables were reported as mean ± standard deviation or median. Categorical variables were expressed as frequencies (%). The difference in the distribution of variables was checked using Chi square/Fishers exact test as appropriate. A p value ≤ 0.05 was used as the criterion for statistical significance. All data generated in the study were analyzed using the SPSS software - version 16.0.

RESULTS

1. Demographic profile

A total of 57 patients were included in the study. Majority of the patients were from Bangladesh (14/57, 24.6%) followed by Tamil Nadu (12/57, 21.1%), Jharkhand (9/57, 15.8%) and West Bengal (7/57, 12.3%) The remaining patients were from other Indian states and Nepal as shown in table below.

Table 4.Region wise distribution of study patients

Region	Number of cases	Percentage (%)
Bangladesh	14	24.6
Tamilnadu	12	21.1
Jharkhand	9	15.8
West Bengal	7	12.3
Andhra Pradesh	3	5.3
Bihar	3	5.3
Nepal	3	5.3
Tripura	2	3.5
Uttar Pradesh	1	1.8
Manipur	1	1.8
Kerala	1	1.8
Karnataka	1	1.8
Total	57	100

Baseline characteristics

The number of male patients (n=38) was double the females (n=19). The ages of the patients ranged from 24-64 years, median age being 47 years. The median weight was 62 kg and median BMI was 25. Baseline ALT levels varied from 10 to 271 U/ml, median being 70 U/ml. The distribution of baseline ALT levels (3 × upper limit of normal) was not different in patients with low vs. high viral loads (≤600,000 vs. >600,000 IU/ml).

2. Distribution of HCV genotypes

Of the 57 patients, 12 (21%) were infected with HCV genotype 1, 43 (75%) with genotype 3 and 2 (4%) with genotype 4.

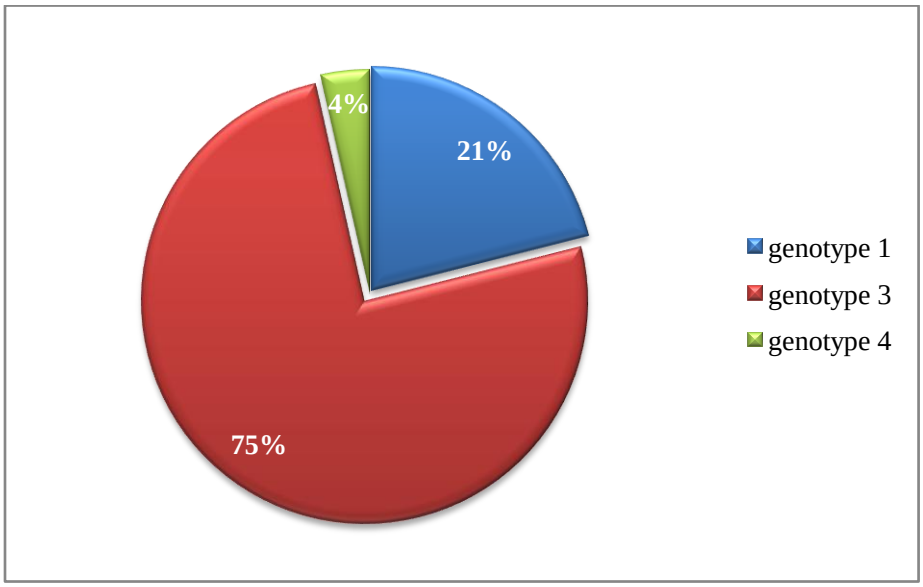
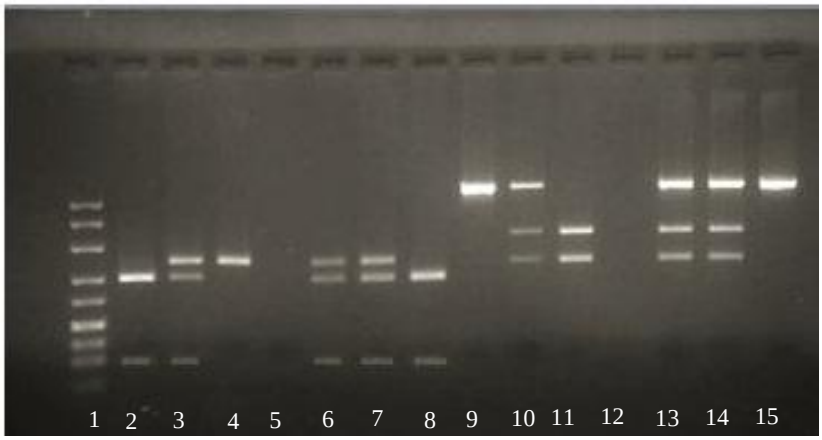


Figure 10.Distribution of HCV genotypes in the cases

3. IL28B genotyping analysis

Host genotyping was done on 57 patients to determine the polymorphisms at loci rs12979860 and rs8099917 using PCR-RFLP as described previously. The polymorphisms were identified based on the lengths of the bands obtained on digestion of the amplification products.



Lane 1: ladder (25 to 500 bp)

Lanes 2 to 8: rs12979860

Lane 2, 8: CC Lanes 3, 6, 7: CT Lane 4: TT

Lanes 9 to 15: rs8099917

Lane 9,15: TT Lane 10,13,14: GT Lane 11:GG

Lanes 5 and 12: Negative control

Figure 11.Documentation under UV light following RFLP

A subset of the samples were bidirectionally sequenced for verification of RFLP results. The figures below depict electropherograms representing the possible genotypes of the two polymorphisms.

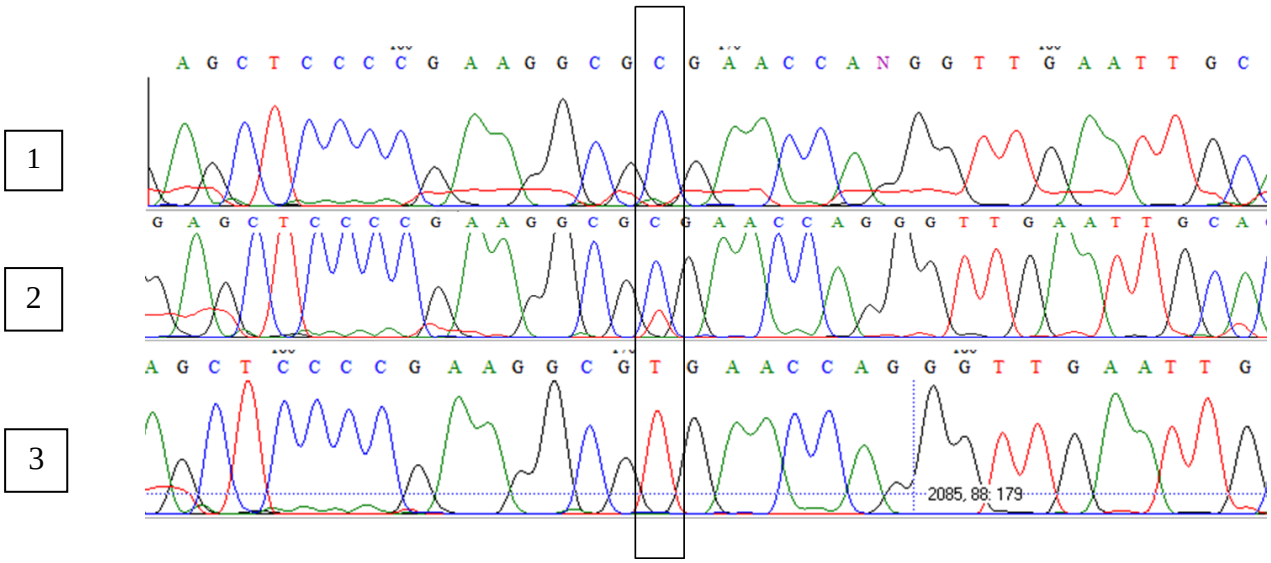


Figure 12.Chromatogram showing sequence of rs12979860 (C/T) of IL28B gene.

Lanes 1–3 show CC, CT, and TT host genotypes in that order

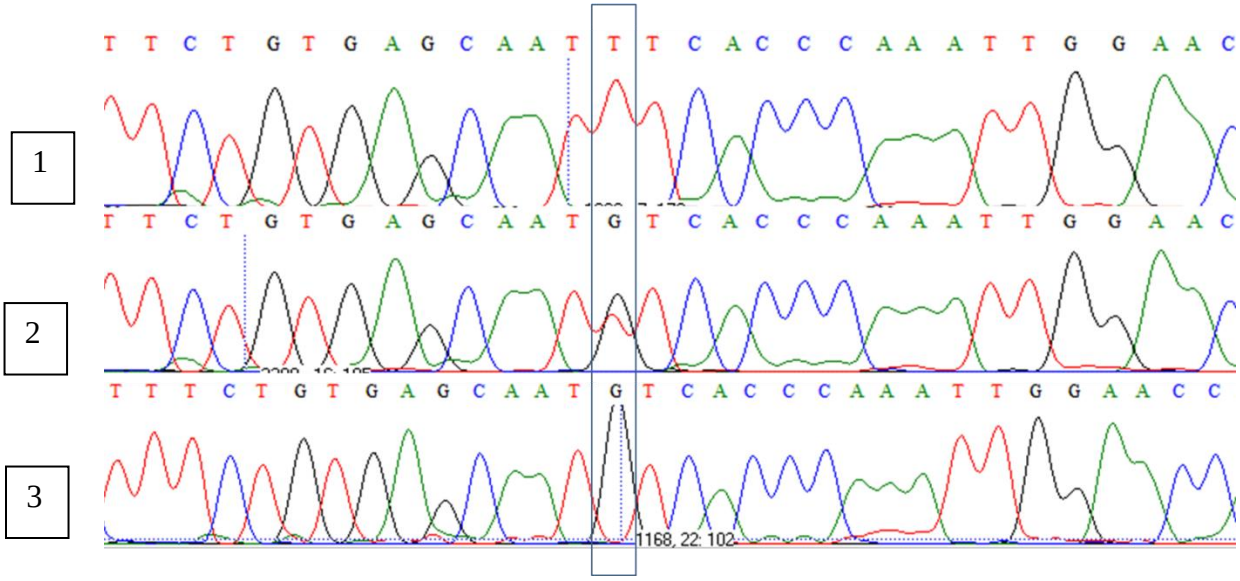


Figure 13.Chromatogram showing sequence of rs8099917 (T/G) of IL28B gene.

Lanes 1–3 show TT, GT, and GG host genotypes in that order

4. Frequency distribution of IL28B polymorphisms in the cases

The frequency of CC genotype at rs1297960 was found to be 59.65 % (34 out of 57 individuals), compared to CT at 33.33 % (19 out of 57). Genotype TT was found in 7.02 % cases (4 out of 57).

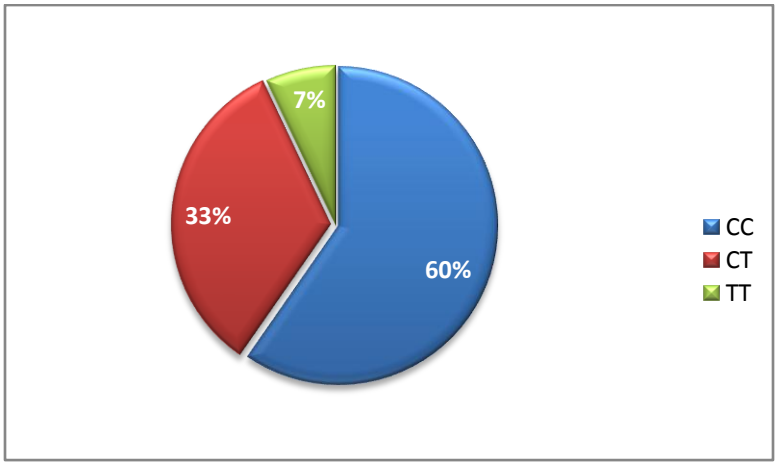


Figure 14.Frequency distribution of rs1297960 polymorphism in cases

On analysis of host genotype at rs8099917 it was found that TT was the commonest, present in 71.93% individuals (41 out of 57), followed by GT in 22.81% cases (13 out of 57) and then GG, in 5.26% individuals (3 out of 57).

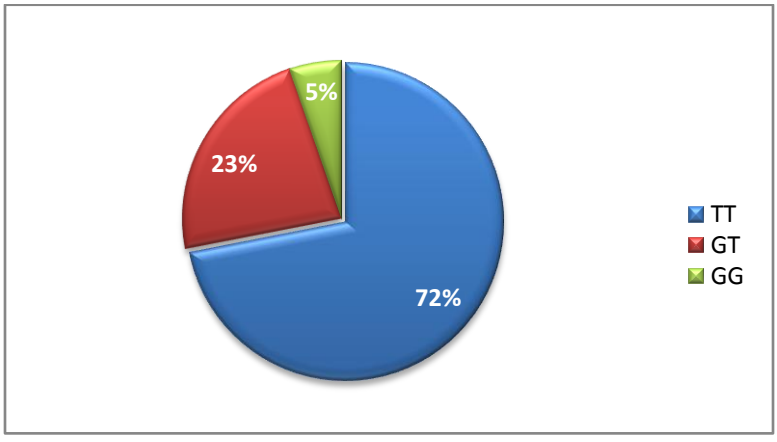


Figure 15.Frequency distribution of rs8099917 polymorphism in cases

Frequency distribution of IL28B polymorphisms in HCV genotype 1, 3 and 4 infected patients

The distribution of the various genotypes of the two polymorphisms were analysed separately in HCV genotype 1, 3 and 4 infected patients, as is depicted in the figures below.

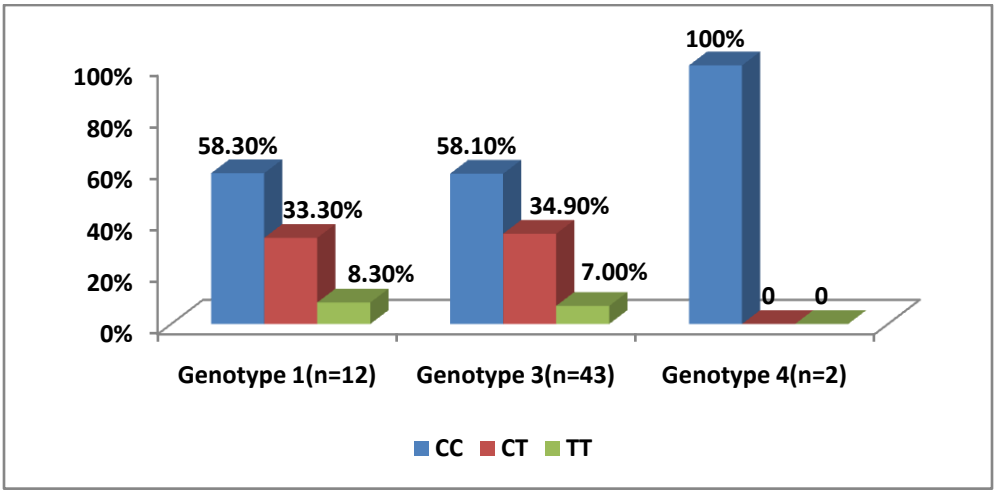


Figure 16.Frequency distribution of rs1297960 polymorphism in HCV genotype 1, 3 and 4 infected patients.

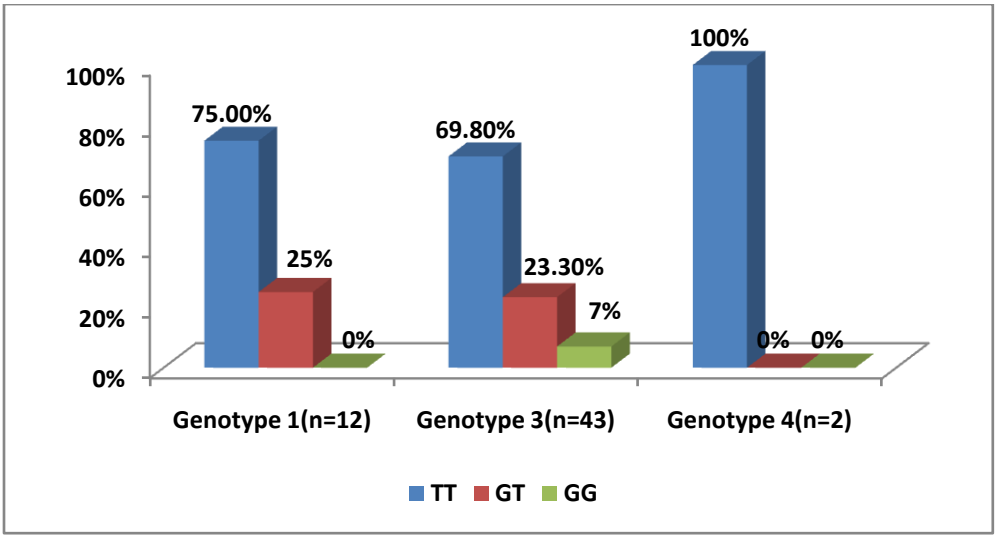


Figure 17.Frequency distribution of rs8099917 polymorphism in HCV genotype 1, 3 and 4 infected patients

5. Follow up of the study patients

The viral load in the patients were monitored at 4 weeks (RVR), 12 weeks (EVR), 24/48 weeks depending upon the genotype (ETR) and six months after end of treatment (Sustained viral response, SVR). Of the 57 recruited patients, 34 completed follow up till measurement of SVR during the course of the study (genotype 1=7, genotype 3=26 and genotype 4=1). The remaining 23 cases were at different time points of follow up. The RVR, EVR, ETR and SVR rates were calculated for both genotypes 1 and 3, and are depicted in the figure below.

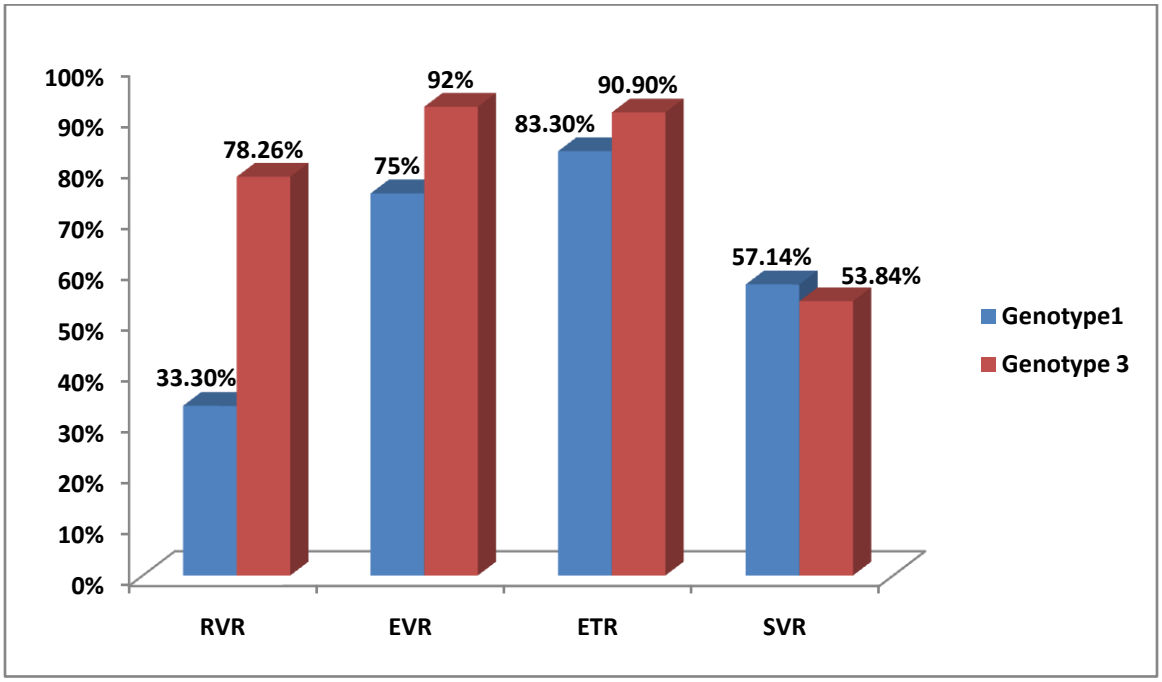


Figure 18.Virological response at different time points in genotype 1 and 3 infected patients

RVR Rapid virological response, EVR Early virological response, ETR End of treatment response, SVR Sustained virological response

Response was defined by achieving sustained lack of viraemia six months after completion of treatment, ie, SVR. Of the 34 patients who had completed follow up till the time point of SVR during the course of this study, 19 achieved SVR while 15 did not, and included both non-responders and relapsers. Both non-responders and relapsers have been grouped together and referred to as non-responders in the analysis.

The overall response rate was 55.9%. 4 out of 7 genotype 1 infected patients were responders (response rate = 57.1%). Similarly, 14 out of 26 genotype 3 infected patients were responders (response rate = 53.8%), showing no difference (P value =1.0).

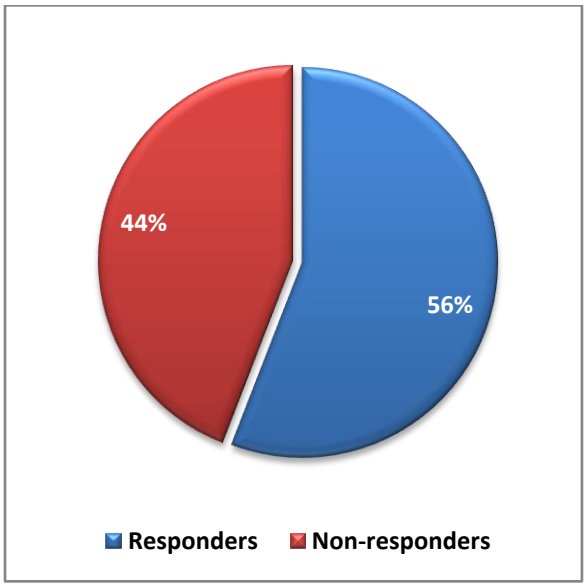


Figure 19.Rates of response (n=34)

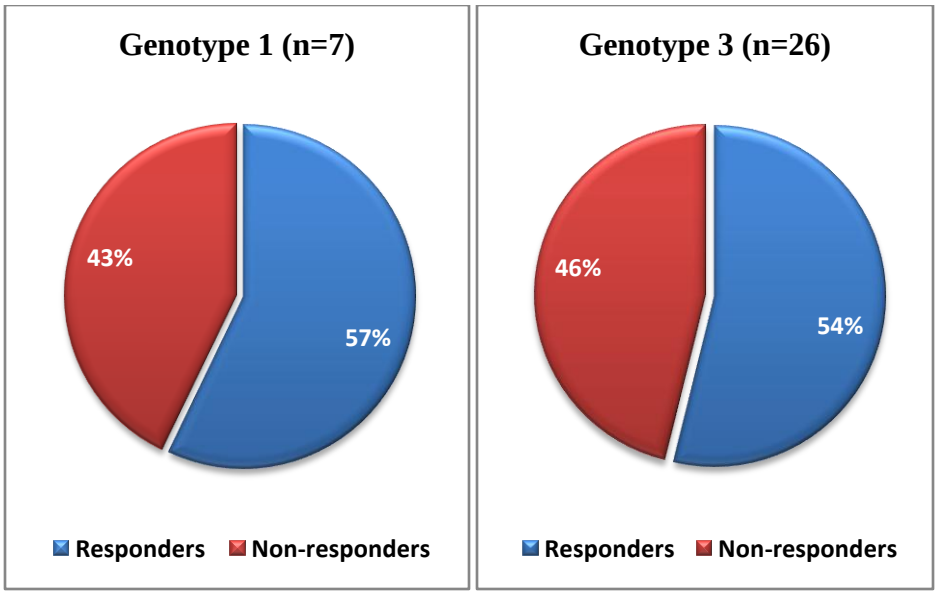


Figure 20.Rates of response in HCV genotypes 1 and 3 infected patients

Viral load at the various treatment milestones were plotted separately for non-responders (figure 21) and responders (figure 23). Pre-treatment viral loads varied from $7.2x10^3$ to $7x10^6$ IU/ml, median being $3.8x10^5$ IU/ml. Most of the treatment failure cases showed undetectable viral loads when sampled at the time point of EVR and ETR. This was then followed by bouncing back of HCV RNA (virological breakthrough) after cessation of therapy. Contrastingly, none of the treatment responders showed detectable viral load at any point of time (figure 22).

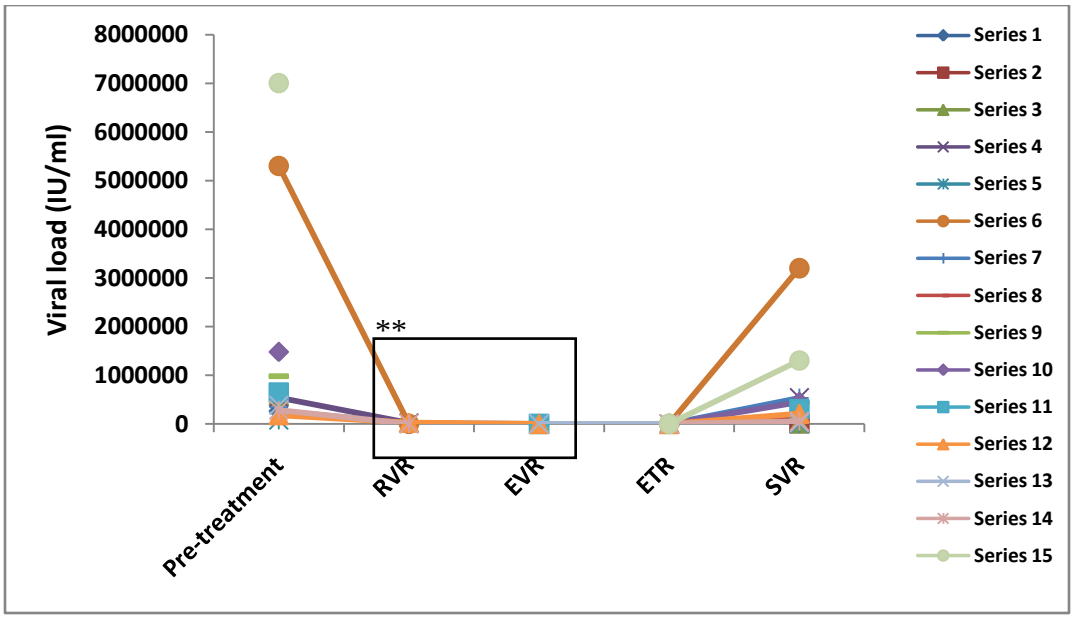


Figure 21A.Viral load monitoring at various time points in treatment non-responders (n=15) **Viral load values at time points of RVR and EVR have been plotted again below, in order to depict values at an appropriate scale.

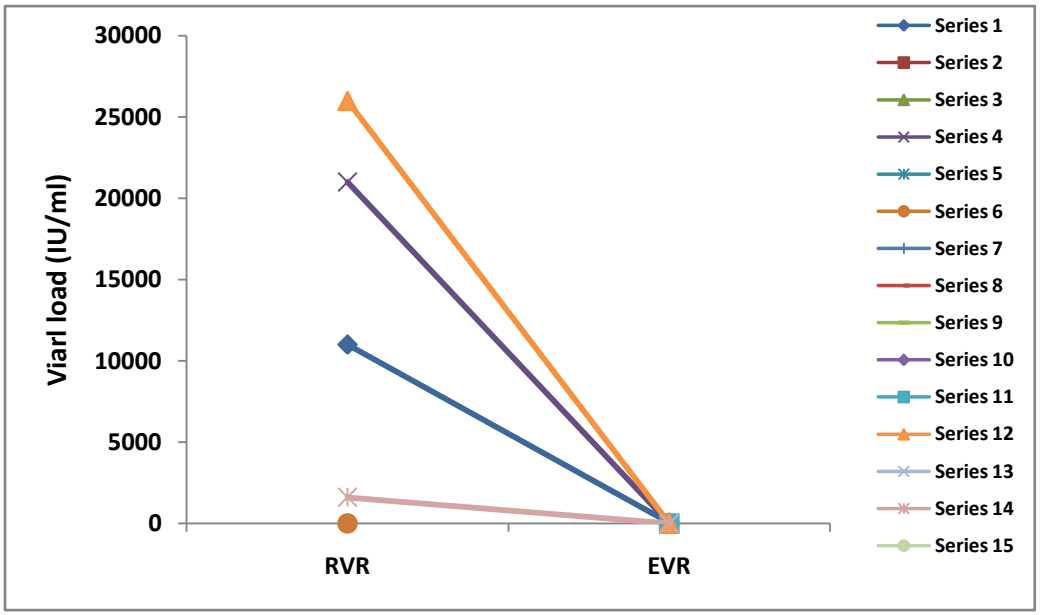


Figure21B.Viral load monitoring at time points of RVR and EVR in treatment non-responders (n=15)

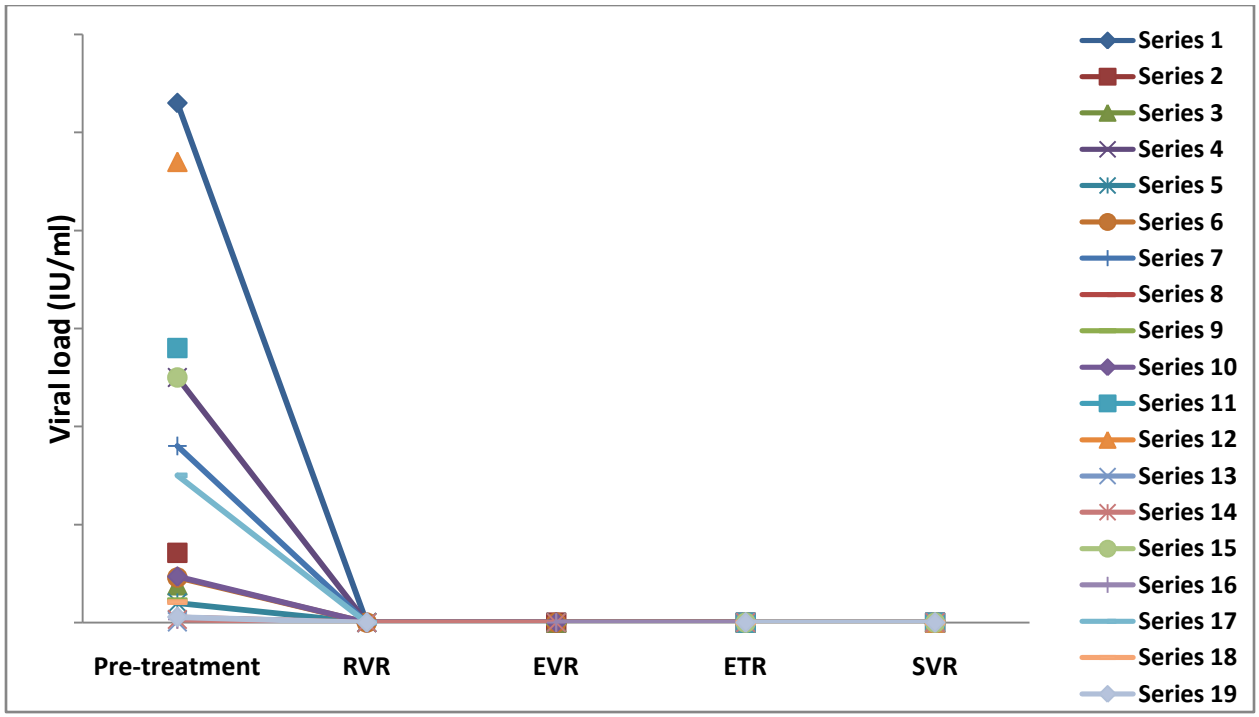


Figure 22.Viral load monitoring at various time points in treatment responders (n=19)

All patients had undetectable viral load at all time points they were sampled, thus yielding flat lines in graph.

6. IL28B frequency distribution in responders and non-responders

Distribution of rs12979860 genotypes

The frequency distribution of rs12979860 CC/CT/TT genotypes in responders (n=19) was 84.2%, 10.5% and 5.3% respectively. On the other hand the distribution in non responders (n=15) was 40%, 53.33% and 6.66% for respectively. A comparative analysis of the distribution of these genotypes in responders and non-responders has been depicted in the figure below.

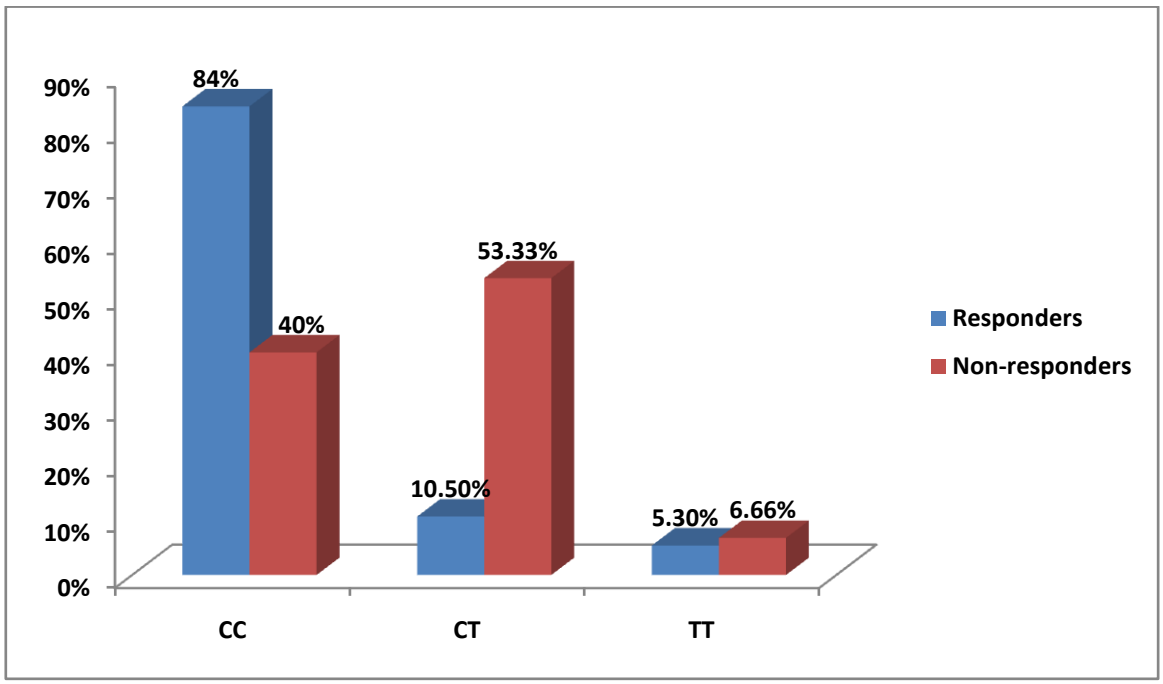


Figure23. Frequency distribution of rs12979860 genotypes in responders and non-responders

To analyse the impact of the 'C' allele on treatment response, genotypes CT and TT were grouped together as non-CC. The distribution of CC vs. non-CC was compared in responders and non-responders. The non-CC genotypes were found to be significantly higher in non-responders (60%) compared to responders (15.8%), with a p value of 0.012, as is depicted in the figure below.

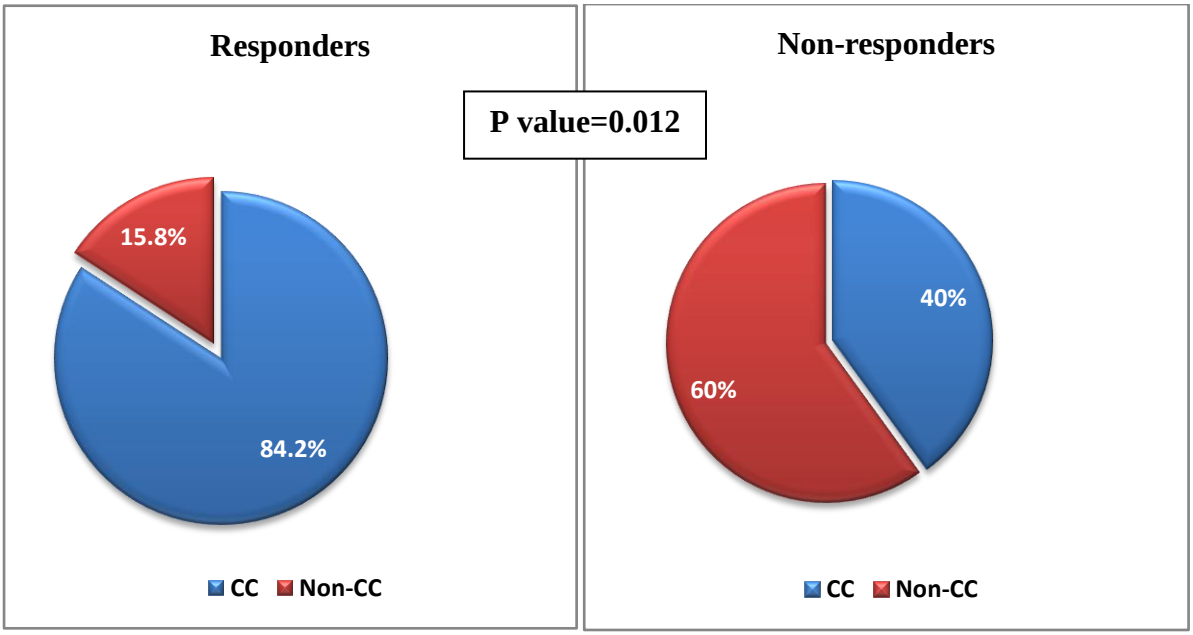


Figure 24.Distribution of the rs12979860 CC and non-CC genotypes in responders and non-responders

Distribution of rs8099917 genotypes

A similar analysis was done for rs8099917 polymorphism. The frequency distribution of rs8099917 TT/GT/GG genotypes in responders (n=19) was 84.2%, 10.5% and 5.3% respectively. The distribution in non responders (n=15) was 53.33%, 40%, and 6.66% for respectively. The same has been represented in the following figure.

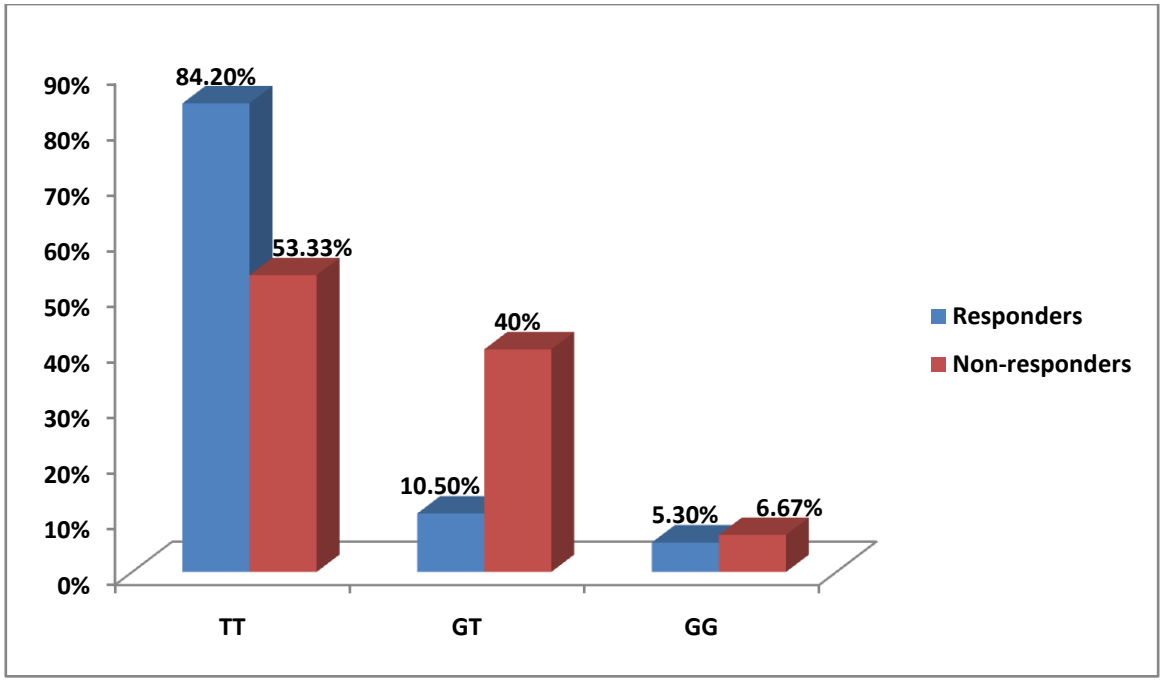


Figure 25.Frequency distribution of rs8099917 genotypes in responders and non-responders

GT and GG were grouped together as non-TT. Non-TT genotypes were higher in non-responders (46.67%) compared to responders (15.80%), although the difference was not significant (p value= 0.068), but showed a trend (figure 26).

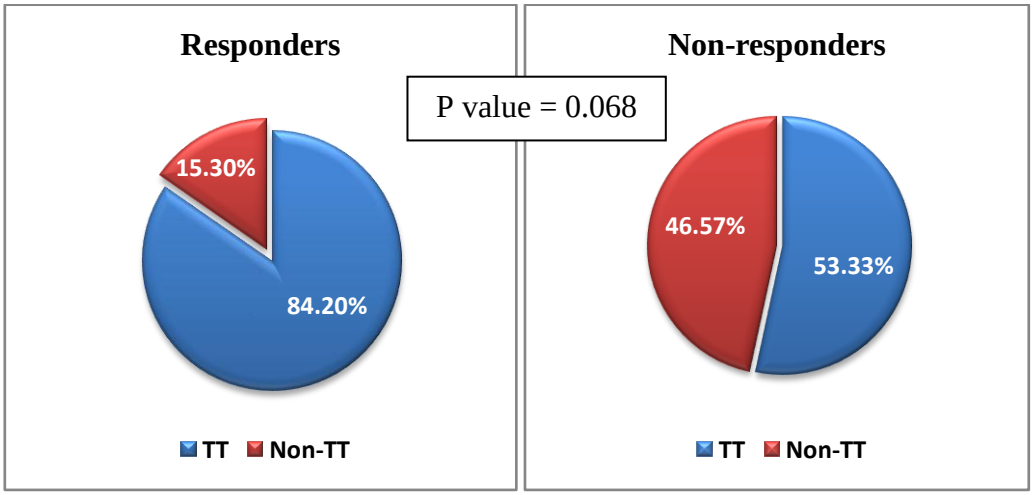


Figure 26.Distribution of rs8099917 TT and non-TT genotypes in responders and non-responders

7. Analysis of the effect of other variables on response to treatment

Other variables were analyzed for their effects on SVR. Of them, RVR was the only factor found to be a determinant of SVR.

A. Age

When study participants ≤40 years vs. >40 years of age were compared for the response to anti-viral therapy, there was no difference between the two age groups (p value=0.426)

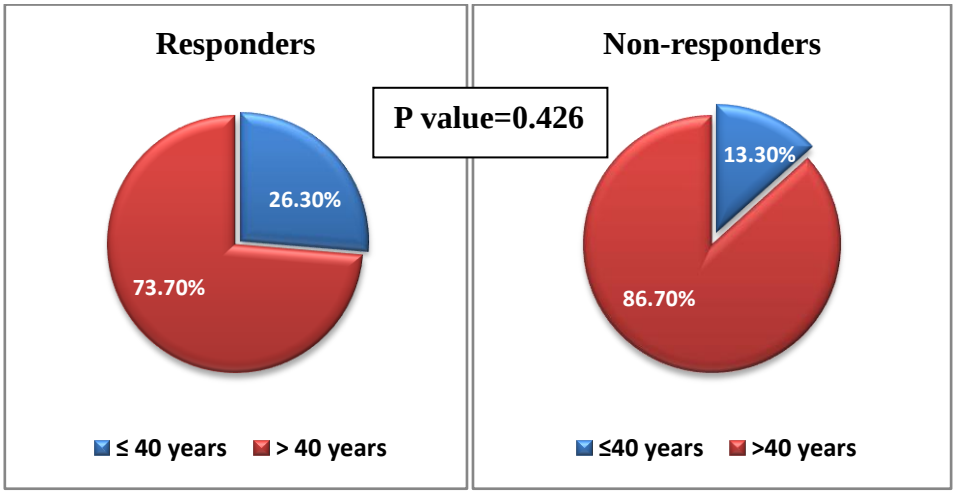


Figure 27.Distribution of age (≤40 years vs. >40 years) in responders and non responders

B. Gender

Association of the gender of the study participants with treatment response was analyzed. However, no difference was found between males and females (p value=0.128) (figure 28)

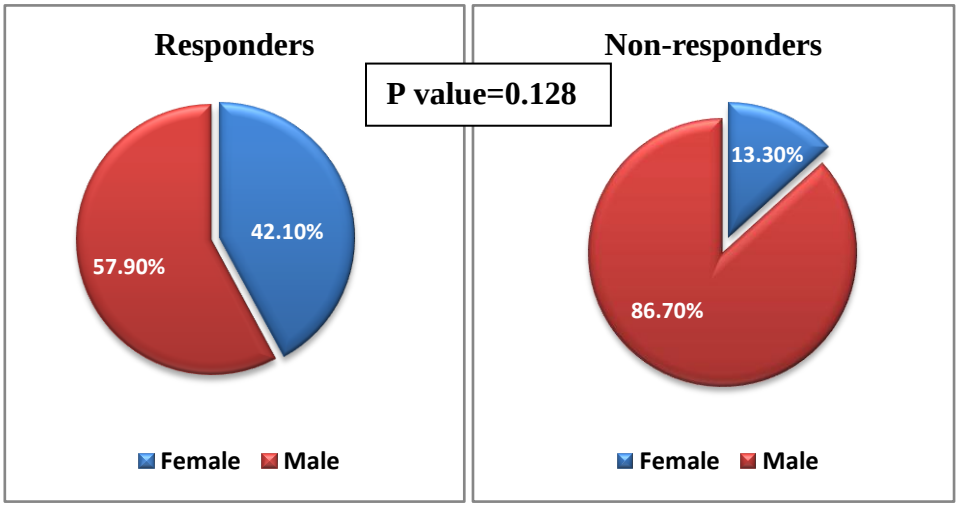


Figure 28.Distribution of gender in responders and non-responders

C. Body mass index

Patients were grouped into two categories based on their BMI (≤ 25 vs. >25). However, a lower BMI was not found to be associated with SVR (figure 29).

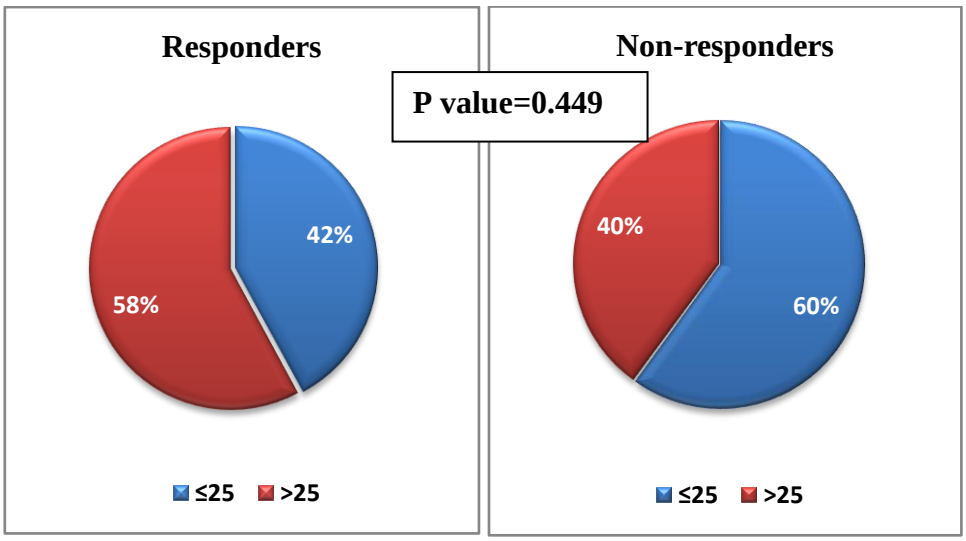


Figure 29.Distribution of body mass index in responders and non-responders

D. Rapid viral response (RVR)

RVR refers to undetectable viral load at 4 weeks of initiation of interferon based therapy. Of the 34 patients analysed, 14 had a viral load measurement at the time point of RVR. Of the 14 patients tested, 4 had detectable viral load, ie, did not attain RVR. All of these 4 patients turned not to be non-responders when followed up till the time point of SVR. On the other hand, of the 10 patients who showed undetectable HCV RNA levels at week 4 (RVR attained), 9 attained SVR, while 1 did not. Attainment of RVR was found to be a significant factor associated with response to treatment (P value=0.005).

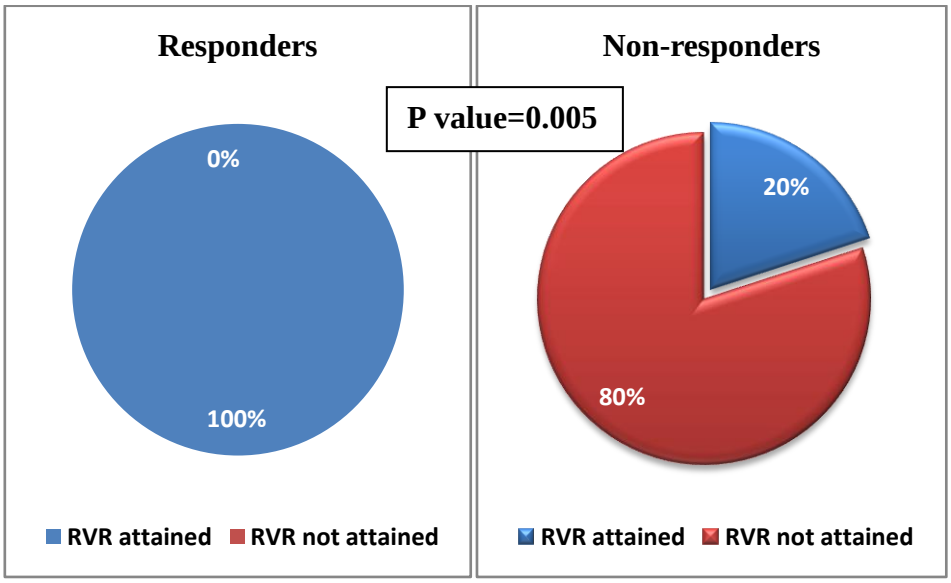


Figure 30.Distribution of RVR attained vs. not attained in responders and non-responders (RVR Rapid viral response)

E. Pre-treatment viral load

Viral load before initiation of treatment was classified as low and high (≤600,000 vs. >600,000 IU/ml) and was analysed for its impact on response. There was no difference in the distribution of viral loads in responders and non-responders (P value=0.710).

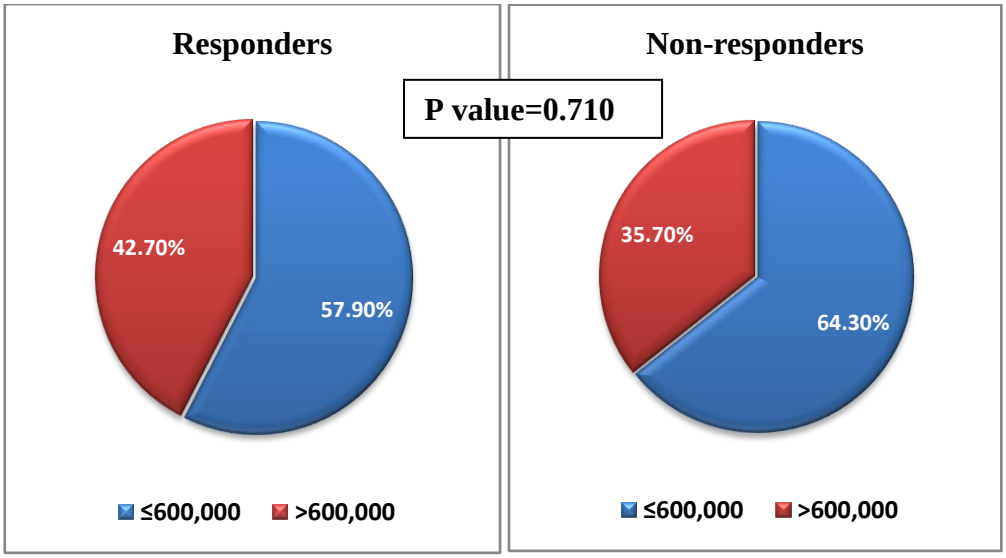


Figure 31.Distribution of pre-treatment viral load (≤600,000 vs. >600,000) in responders and non-responders

F. Treatment modality

The study cases were on therapy with either PEG-IFN or standard interferon (IFN), in combination with RBV. Whether the treatment modality had a bearing on virological response was looked at. However, no difference was found between the two groups (P value=0.217).

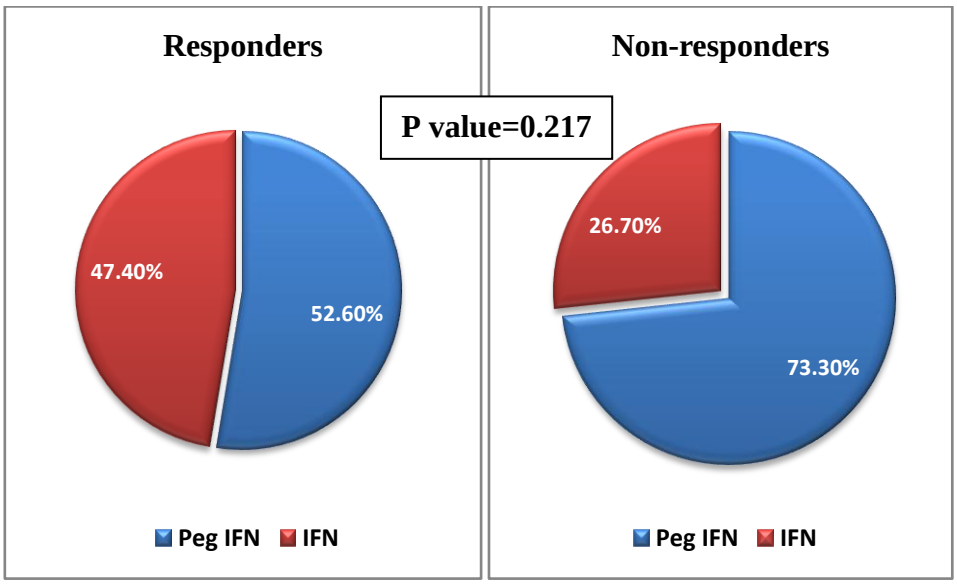


Figure 32.Distribution of treatment modality (PEG-INF vs. IFN) in responders and non-responders

G. Diabetes

When study participants were grouped as diabetics and non-diabetics and compared for the response rates, no difference was found in the two groups

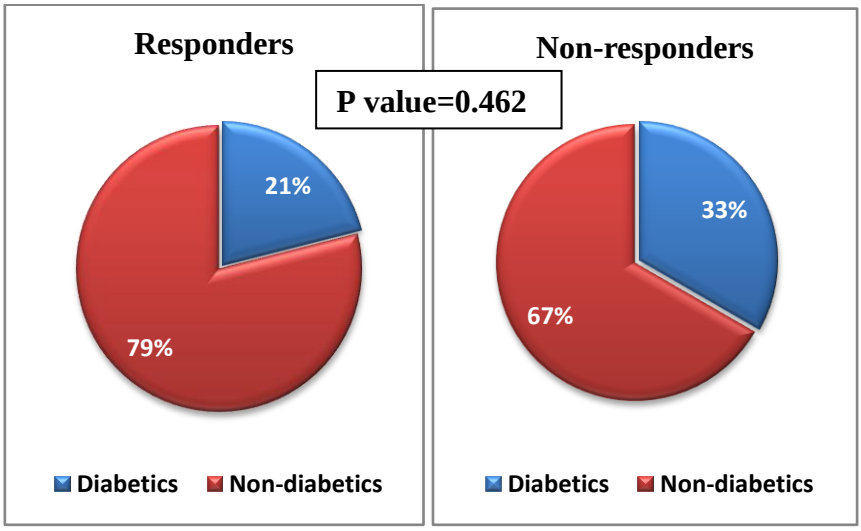


Figure 33.Distribution of diabetics in responders and non-responders

H. Baseline alanine transaminase (ALT) levels

The distribution of pre-treatment ALT levels ≤105 U/ml vs. >105 U/ml (3 × upper limit of normal) was compared in responders and non-responders. The difference was not statistically significant (p value=0.08), though a trend was observed.

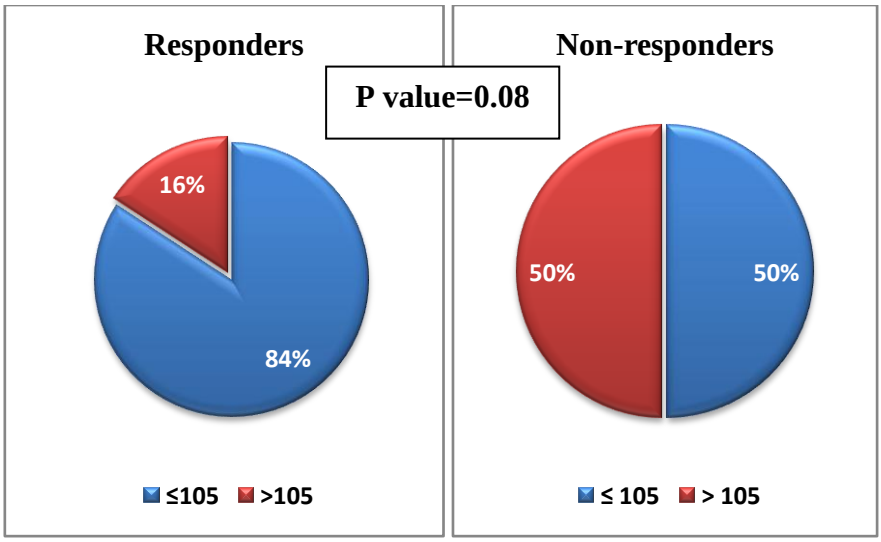


Figure 34.Distribution of ALT levels in responders and non responders

A multivariate analysis was performed for all the variables. However, none of them figured out to be significant in the analysis.

8. Association of the IL28B polymorphisms with RVR

(a) rs 12979860 polymorphism

The distribution of rs 12979860 CC/CT/TT genotypes was studied in individuals who attained RVR and those who did not (figure 35).

The non-CC genotypes were found be much higher in those who did not attain RVR (78%), in comparison to those who did (27%). The difference was statistically significant (P value=0.017), showing that rs12979860 CC genotype was a predictor for RVR (figure 36).

(b) rs8099917 polymorphism

Frequency distribution of rs8099917 TT/GT/GG genotypes was studied in individuals who attained RVR and those who did not. (figure 37)

The non TT genotypes were found in 18% of the individuals who attained RVR and in 56% of individuals who did not attain RVR. However, the difference was not statistically significant, but showed a trend (p=0.077) (figure 38)

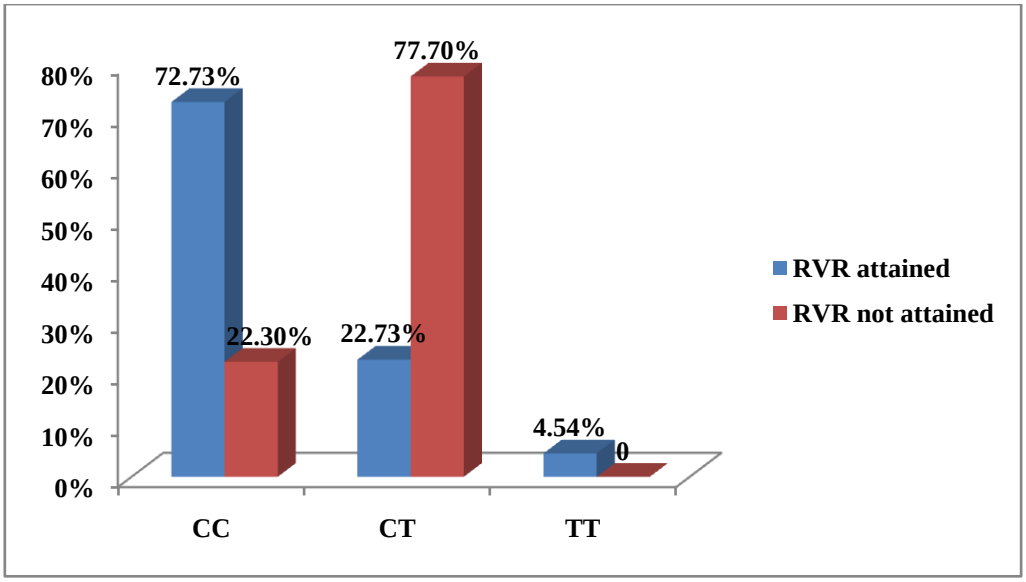


Figure 35.Frequency distribution of rs12979860 genotypes in individuals who attained RVR and in those who did not (RVR Rapid viral response)

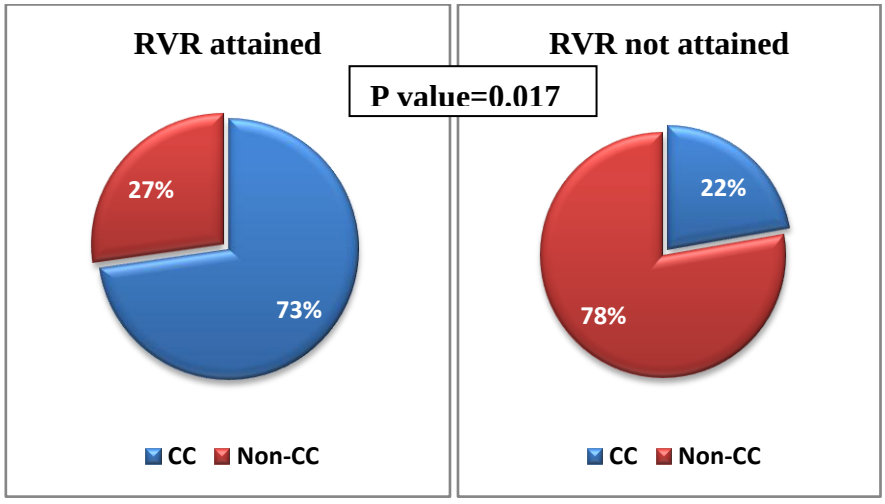


Figure 36.Distribution of the rs12979860 CC and non-CC genotypes in individuals who attained RVR and in those who did not (RVR Rapid viral response)

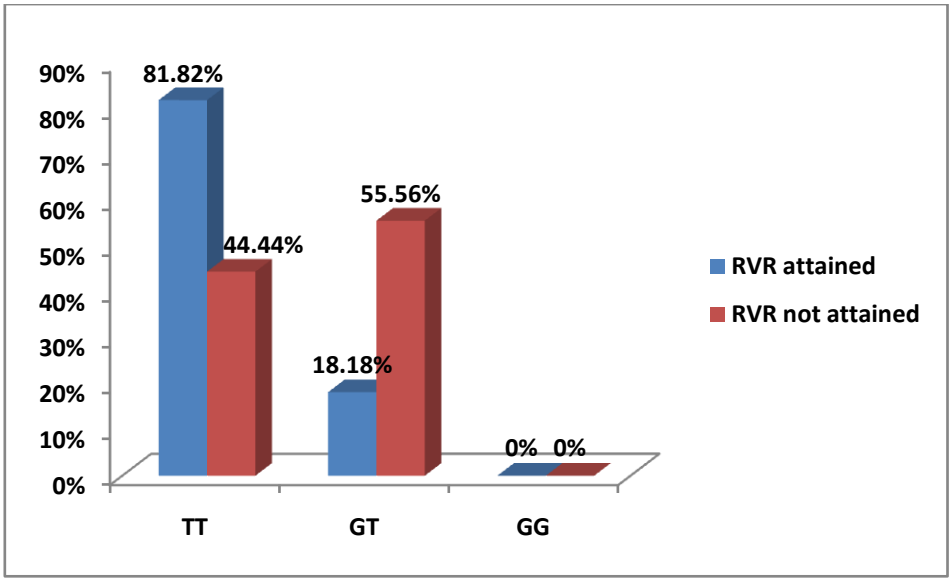


Figure 37.Frequency distribution of rs8099917 genotypes in individuals who attained RVR and in those who did not (RVR Rapid viral response)

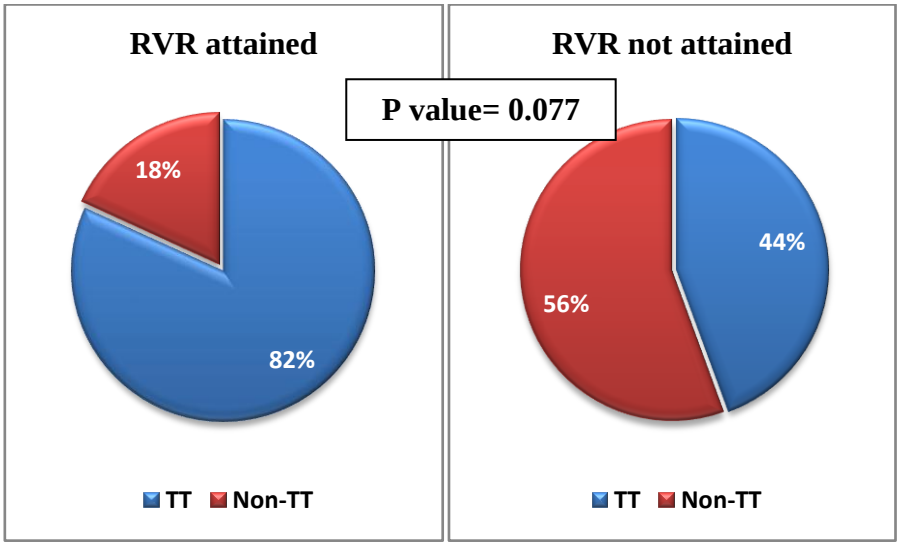


Figure 38.Distribution of the rs8099917 TT and non-TT genotypes in individuals who attained RVR and in those who did not (RVR Rapid viral response)

9. Association of the IL28B polymorphisms with EVR

(a) rs 12979860 polymorphism

The distribution of rs 12979860 CC/CT/TT genotypes was studied in individuals who attained EVR and those who did not (figure 39).

Non-CC genotypes were found in 25% of individuals who did not attain EVR, compared to 39% in those who attained EVR. There was no difference (p=1.00) (figure 40).

(b) rs8099917 polymorphism

Frequency distribution of rs8099917 TT/GT/GG genotypes was studied in individuals who attained EVR and those who did not (figure 41)

Non TT genotypes were found in 29% of individuals who attained EVR and in none who did not attain EVR. The difference was not significant (p value= 0.553) (figure 42)

Thus the IL28B polymorphisms were not found to be associated with EVR.

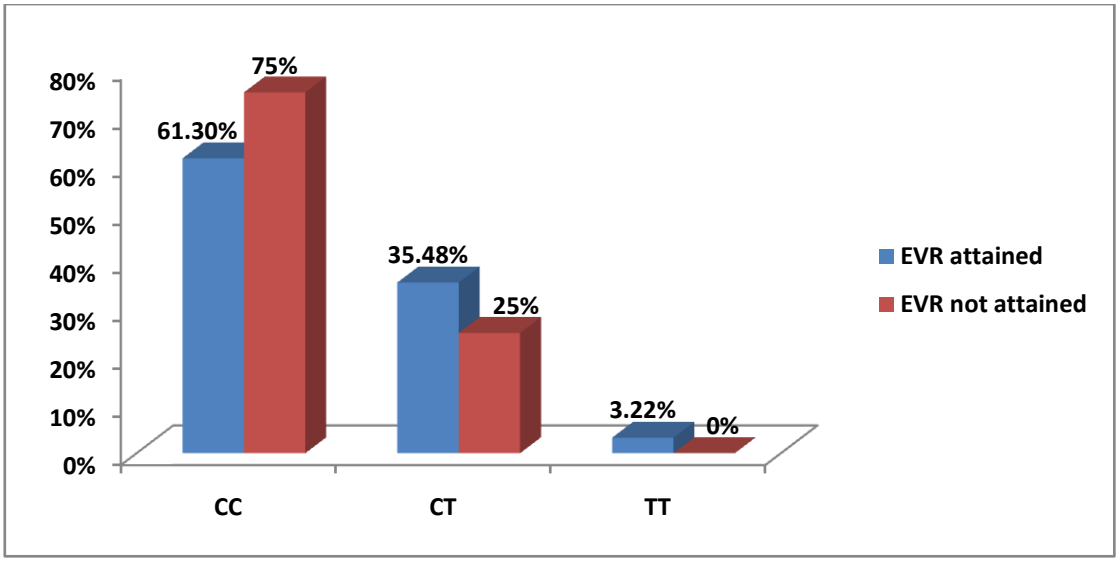


Figure 39.Frequency distribution of rs12979860 genotypes in individuals who attained EVR and in those who did not (EVR Early viral response)

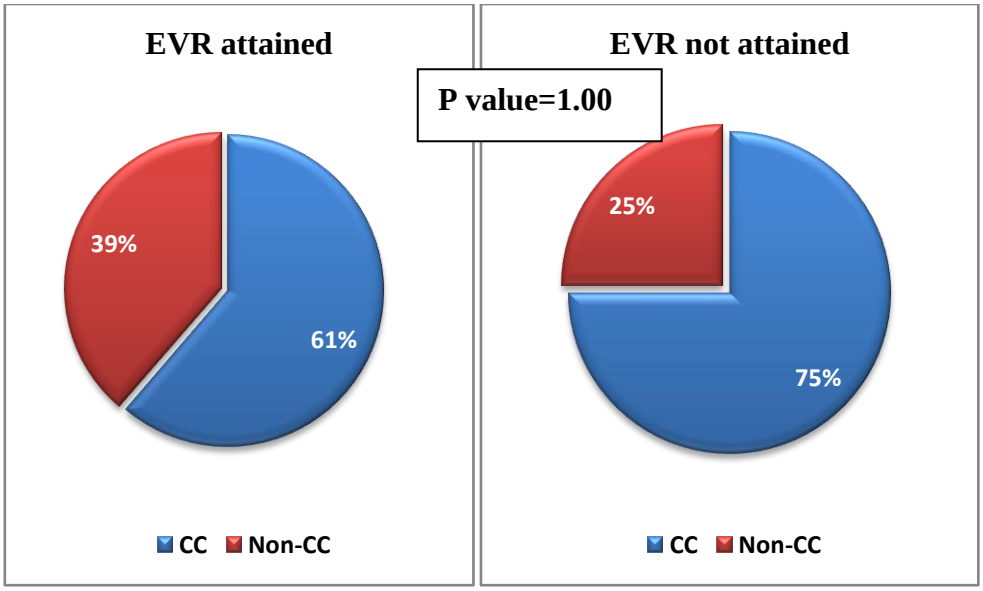


Figure 40.Distribution of the rs12979860 CC and non-CC genotypes in individuals who attained EVR and who did not (EVR Early viral response)

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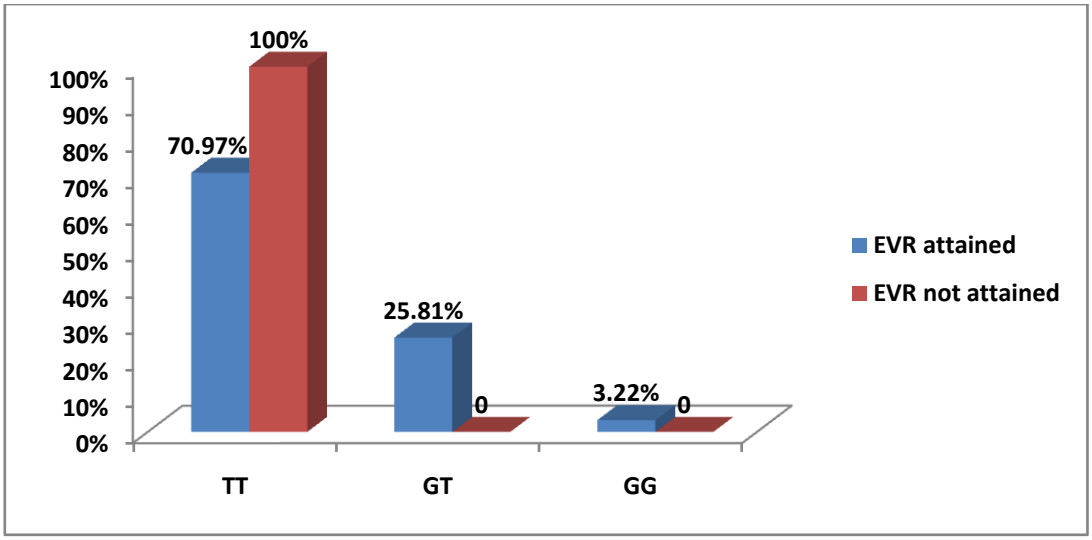

Figure 41.Frequency distribution of rs8099917 genotypes in individuals who attained EVR and in those who did not (EVR Early viral response)

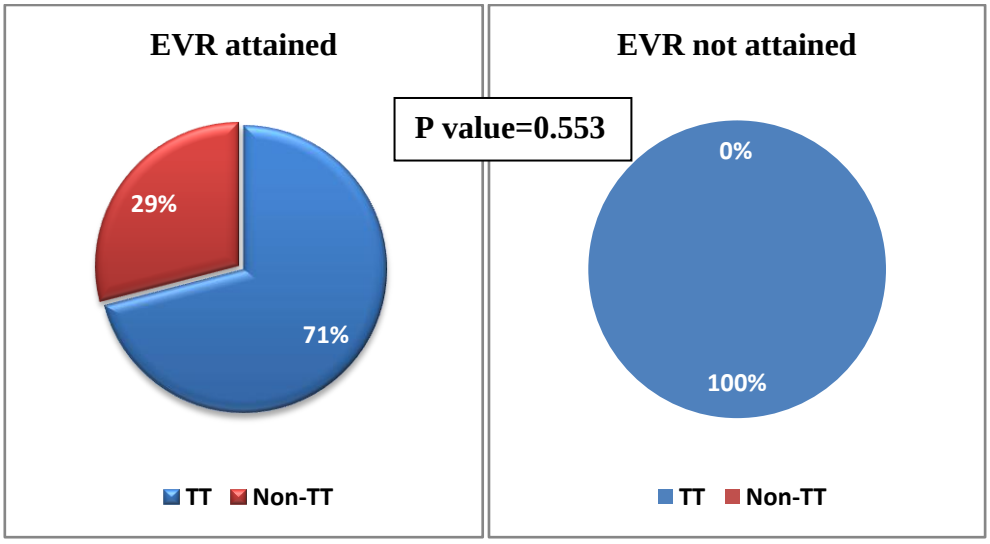

Figure 42.Distribution of the rs8099917 TT and non-TT genotypes in individuals who attained EVR and in those who did not (EVR Early viral response)

10. Association of the IL28B polymorphisms with ETR

(a) rs 12979860 polymorphism

The distribution of rs12979860 CC/CT/TT genotypes was studied in individuals who attained ETR and those who did not (figure 43).

Non-CC genotypes were found in 75% of individuals who did not attain ETR, compared to 40% in those who attained ETR. However, the difference was not significant (p value=0.299) (figure 44).

(b) rs8099917 polymorphism

Frequency distribution of rs8099917 TT/GT/GG genotypes was studied in individuals who attained ETR and those who did not (figure 45).

Non TT genotypes were found in 75% of individuals who did not attain ETR compared to 31% in those who did. The difference was not significant (p value= 0.122) (figure 46).

Thus IL28B polymorphisms were not found to be associated with ETR.

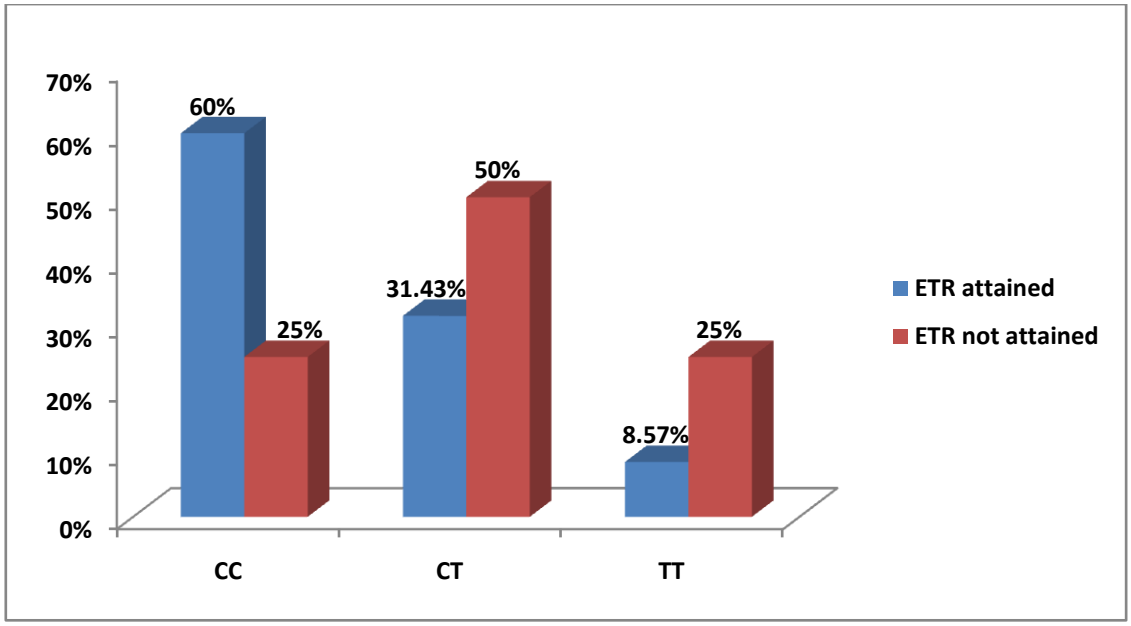

Figure 43.Frequency distribution of rs12979860 genotypes in individuals who attained ETR and in those who did not (ETR End of treatment response)

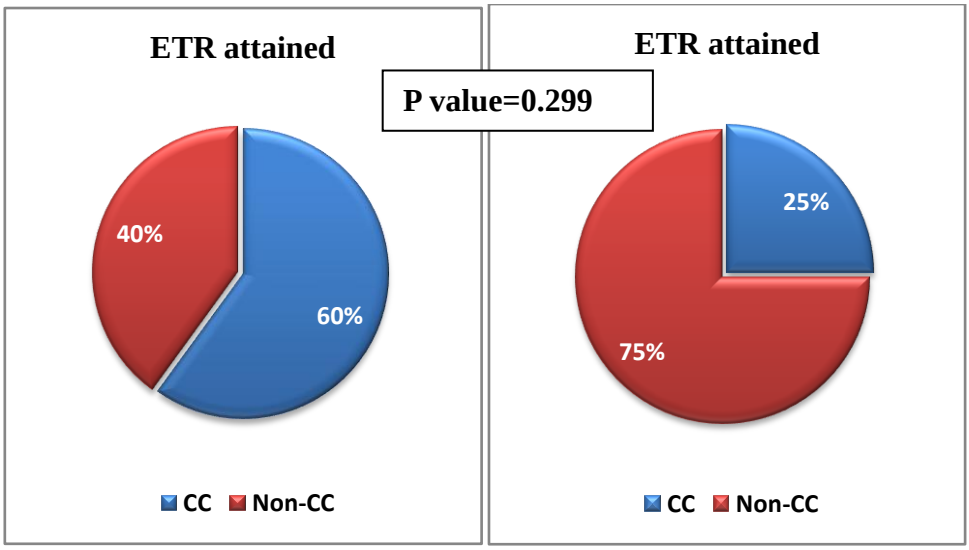

Figure 44.Distribution of the rs12979860 CC and non-CC genotypes in individuals who attained ETR and in those who did not (ETR End of treatment response)

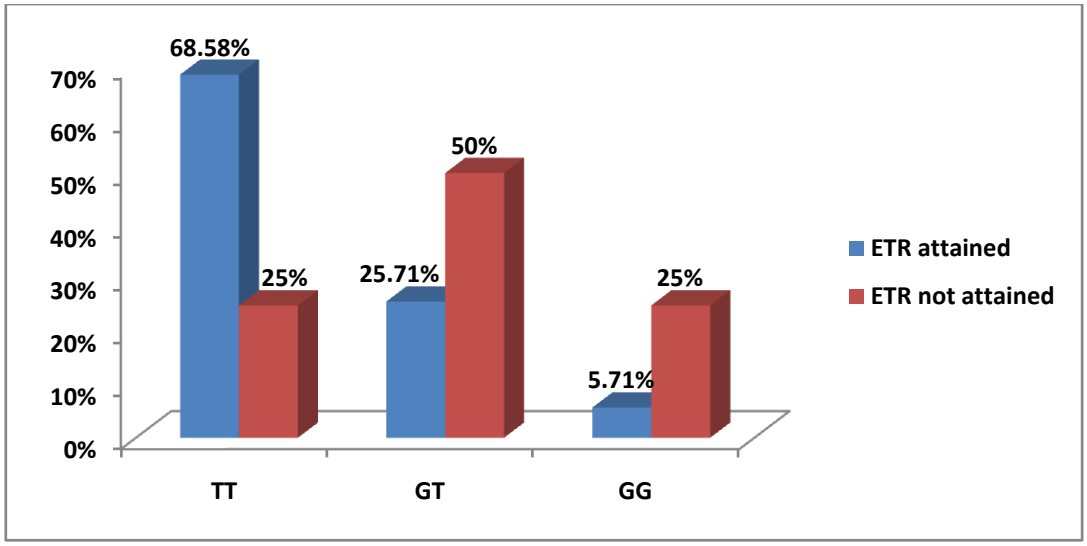

Figure 45.Frequency distribution of rs8099917 genotypes in individuals who attained ETR and in those who did not (ETR End of treatment response)

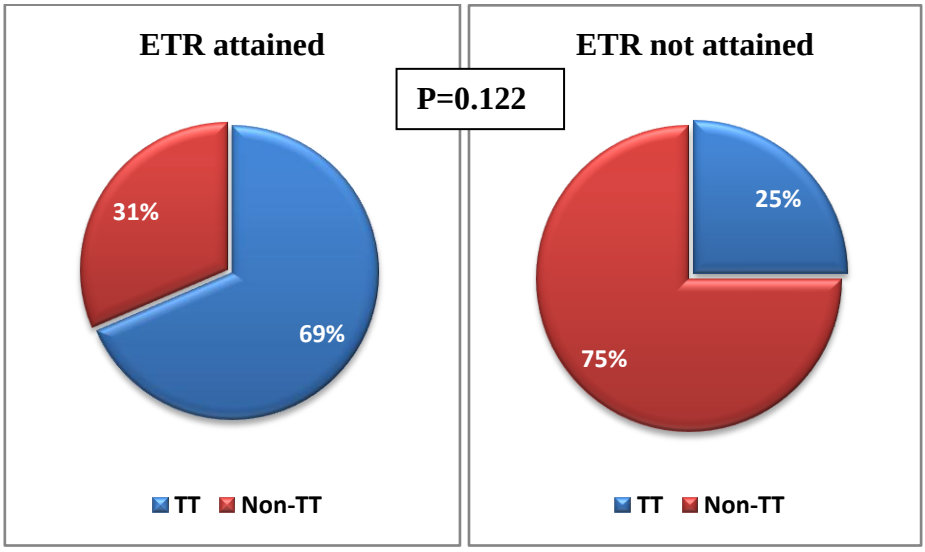

Figure 46.Distribution of the rs8099917 TT and non-TT genotypes in individuals who attained ETR and who did not (ETR End of treatment response)

DISCUSSION

Estimated to infect more than 185 million people worldwide(2) and over 10 million people in India (3), HCV is a common cause of post transfusion hepatitis in resource poor settings and in individuals at risk of unsafe injection practices. Tendency of the virus to cause persistent infection and the plethora of ensuing complications make treatment necessary. Currently, PEG-IFN plus RBV is the standard of care therapy for chronic hepatitis C. However, treatment is expensive and is marred by significant adverse effects, which may be severe enough to lead to withdrawal from treatment. This necessitates serial monitoring of viral load in patients on therapy at specified time points to assess and prognosticate the treatment response. In addition, the significance of viral and host factors which impact on severity of disease and response to treatment becomes immense.

In this study we attempted to determine the frequency and distribution of IL28B gene polymorphisms in patients with chronic HCV infection and their association with response to IFN based treatment. Association of other host and viral factors with treatment response was also looked at.

We recruited 57 HCV genotype 1, 3 and 4 infected patients, who were either on, or had completed IFN based treatment. The study patients hailed from various parts of the Indian subcontinent, including Bangladesh and Nepal in addition to many Indian states. Of the 57 study patients, 12 (21%) were infected with HCV genotype 1, 43 (75%) with genotype 3 and 2 (4%) with genotype 4. Genotype 6 infections were not included in the study owing to their rarity. The distribution of viral genotypes was in concordance with previous two studies from our institution where genotypes 3, 1 and 4 accounted for 64%, 26% and 8%; and 62%, 19% and 6% HCV isolates respectively (7,51).

Assessment of virological response is recommended at specified time points of treatment; RVR at 4 weeks, EVR at 12 weeks, ETR at 24/48 weeks depending upon the genotype and SVR six months after end of treatment. Since ours is a tertiary care referral centre which caters to patients belonging to all parts of the country, the study participants were not available for sampling at all the recommended time points. Suitable treatment milestones were measured whenever possible. A proportion of our study participants (34 out of 57) completed follow up till the time of measurement of SVR during the course of the study, while the remaining 23 cases continue to be at different time points of follow up. A history of compliance to treatment was elicited from all patients, and cases with non-compliance were not included in the study. When analysed for the response rates, genotype 3 and 1 showed similar SVR rates of 54% and 57% respectively.

The response rate of genotype 3 to interferon based therapy (54%) in the present study is lower than that seen in the western part of the world (88, 89), but is similar to the rates reported from our centre previously, when David *et al.* found SVR rate of 57% in HCV genotype 3 infections (124) However, this may be attributable to the referral bias of a tertiary care centre. Interestingly, studies from different parts of India have reported largely variable SVR rates in HCV genotype 3 infections. While high response rate (SVR=70%, n=356) has been seen in North Indian patients (18), a rate similar to ours (SVR=55%, n=83) has been found in a study involving patients from Eastern and North Eastern India (19). A smaller retrospective study from Mumbai, on the other hand, has found a very high response rate (SVR=87.5%, n=24), reflecting the huge heterogeneity in the Indian population (125). In a study involving 38 Asian and 66 Caucasian patients infected with HCV genotype 3, Freshwater *et al*. (126) found significantly lower response rates lower in Asians (42%) compared to Caucasians (62%). Asian patients included in this study were natives of the Indian subcontinent who had acquired infection with genotype 3 in their country of birth and

had later migrated to United Kingdom. These Asian patients had significantly greater fibrosis at presentation, were more frequently diabetic, and had higher liver enzymes at baseline and throughout treatment. Baseline steatosis scores were available for half the patients in both the groups, and showed no difference. Non compliance to treatment was ruled out. However when a multivariate analysis of these factors was performed, nothing turned out to be significant, which the authors ascribed to the small numbers studied. The low response rates seen could be due to longer disease duration and advanced liver disease seen in Asians. On the contrary, SVR rates with genotype 3 infections from other parts of Asia have been much higher; 75% in China, and up to 90% in Korea and Taiwan (127). A multicentric trial comparing response rates in Asians (SVR= 65% n=52) vs whites (SVR=45% n=384) found Asian ethnicity to be highly predictive of viral clearance, irrespective of the infecting HCV genotype (127) .

The response rate (57%) with genotype 1 is a little higher than earlier reported in large multicentric trials across the world (40 to 50%) (86,87). On the other hand, studies from Asia have shown high SVR rates; 61% in Japanese patients, 55 to 70% in Koreans and about 75% in Taiwanese (128). A few studies from India have shown tried to analyze the response rates in HCV genotype 1 infections. While a study from Mumbai (125) found SVR rate in genotype 1 to be 56% (n=16), another report from New Delhi (129) showed similar findings (SVR=55.5%, n=9). A multicentric study (130) involving 12 centres across 8 cities in the country recruited 27 HCV genotype 1 infected cases, of whom 20 were followed up till the time point of measurement of SVR. The response rate was found to be 60% (130) . However, larger studies are warranted to support these findings.

Ever since four independent genome-wide association studies identified single nucleotide polymorphisms (SNPs) near the IL28B gene to be strong genetic determinants of treatment induced clearance of HCV (10–12,104), a multitude of studies have attempted to establish the

association. The two polymorphisms most strongly associated with treatment response are rs12979860 (C/C, C/T and T/T) and rs8099917 (T/T, T/G and G/G). The C/C genotype at rs12979860 and T/T genotype at rs8099917 have been shown to be associated with a good treatment response. While the impact of these polymorphisms on genotype 1 and 4 infections has been strongly established, their association with genotype 3 is considered weak (16). It is suggested that the favourable alleles C/C at rs12979860 and T/T genotype at rs8099917 are associated with lower intrahepatic levels of Interferon stimulated genes (ISGs) (106). Individuals with upregulated ISGs prior to treatment show poor outcome with Interferon based therapy, because the already induced ISGs fail to respond to exogenous interferon supplementation due to the saturated signalling pathways (131). Although an association between these polymorphisms and ISG levels has been seen, the exact mechanistic link is yet to be elucidated. Not only have these polymorphisms been linked with response to treatment, but their association with spontaneous clearance of the infection has also been described (14). The favourable genotypes at these loci have been found to be predictive of SVR in individuals who do not achieve RVR (132). The relevance of IL28B polymorphisms in the era of direct acting antivirals (DAA) is yet to be defined. Addition of DAA to interferon based regimens improves on-treatment viral kinetics, thus attenuating the impact of the SNPs, which also modulate on-treatment viral kinetics (133). The role of these polymorphisms in interferon free regimens is under investigation, while some preliminary reports show greater success rates with favourable IL28B genotypes (134).

Genotyping of the 57 study participants was done to determine the polymorphisms at loci rs12979860 and rs8099917. The frequency distribution of rs12979860 CC/CT/TT genotypes was found to be 60%, 33% and 7% respectively. The distribution pattern we found was comparable to the previous studies from the country (17,18,19). Sivaprasad *et al.* (17) in their study on 220 uninfected individuals from Andhra Pradesh found the CC/CT/TT genotype

distribution to be 59%, 34% and 7% respectively. Gupta *et al.* (18) from New Delhi analysed the rs12979860 SNP in 356 HCV genotype 3 infected patients and 360 matched healthy controls. The CC/CT/TT genotype distribution was found to be 58%, 35%, and 7% respectively in cases of hepatitis C and 61%, 35%, and 4% respectively in uninfected controls, the difference not being significant. In another study by Firdaus *et al.* (19) involving 400 HCV seropositive patients from Eastern and North Eastern India and 100 unrelated healthy controls, there was a greater distribution of the CC allele at the rs12979860 locus in both the groups. The frequency of CC/CT/TT genotypes was found to be 71%, 24% and 5% in cases, and 73%, 23% and 4% in controls respectively. All these studies have shown a much higher prevalence of CC allele at rs12979860 in the Indian subcontinent, when compared to the distribution in other ethnicities. In a landmark multicentric genome wide association study (111), the frequency distribution of CC/CT/TT genotypes was found to be 37%, 51% and 12% respectively in Caucasians, 14%, 49% and 37% respectively in African-Americans, 29%, 48% and 22% respectively in Hispanics. This stark difference in distribution of the SNP in various ethnicities is believed to partially account for the large differences seen in response rates in different populations (10), with Asians responding best to interferon based treatment, followed by Caucasians and then African Americans (97).

On analysis of host genotype at rs8099917, we found that TT was the most common, present in 72% individuals, followed by GT in 23% cases and then GG in 5% individuals. The distribution pattern observed by us was similar to that seen by Firdaus *et al.* (19). They had found TT/GT/GG genotype frequency to be 77.50%, 15% and 7.50% respectively in HCV seropositive cases. In the control group genotype, TT was found in 70% individuals, followed by GT in 30%. There were no GG alleles in this group (19). There are no other studies from India determining the frequency distribution of this polymorphism. A large genome wide association study involving 1015 Caucasian chronic hepatitis C patients found the TT/GT/GG

distribution to be 58%, 37% and 5% respectively(104). In sharp contrast, in a study from Taiwan, the TT genotype was found in 85% of the cases, GT in 15% and GG in none (135). This suggests the prevalence of the TT genotype in Indians (72-77%) to be intermediate between Caucasians (58%) and East Asians (85%), which again mirrors the comparative rates of response seen in these populations.

To analyze the impact of the two polymorphisms on response to treatment, we compared the distribution of the rs12979860 CC/CT/TT and rs8099917 TT/GT/GG genotypes in responders and non-responders. Both non-responders and relapsers were grouped together as non-responders for the sake of the analysis. The CC genotype at rs12979860 was observed in 84% of responders compared to only 40% in the non-responders, suggesting that the polymorphism is significantly associated with SVR. On analysis of the rs8099917 polymorphisms, TT genotype was found in 84% of those who attained SVR compared to 53% in those who did not. Though the difference between the two groups was not statistically significant, a trend was seen. The impact of these polymorphisms on RVR, EVR and ETR was also studied. No association of the SNPs was seen with EVR and ETR. However, CC genotype at rs12979860 was significantly associated with RVR, and TT genotype at rs8099917 again showed a trend. Thus we inferred that rs12979860 polymorphism influences treatment response in chronic hepatitis C patients and the CC genotype is a predictor of RVR and SVR. The association with rs8099917 polymorphism could not be established, though there was a trend.

Although the association of these polymorphisms with genotype 1 HCV has been documented in several studies from many regions of the globe (109,110), the association with genotype 3 has been controversial, with studies yielding contrasting results. Comparing our results with the available Indian literature, our findings reiterated the conclusions of Gupta *et al.* (18) and Firdaus *et al.* (19) with regard to the rs12979860 polymorphisms. However, we

did not find a significant association of the rs8099917 polymorphism with response to treatment, unlike Firdaus *et al.*(19). The limited numbers analyzed in our study (n=34) might be a reason for masking of the association.

We also attempted to analyze the association of other host and viral factors on SVR. Patients were categorised based on age (≤40 years vs. >40 years), gender, body mass index (≤ 25 vs. >25), diabetic status, pre-treatment viral load (≤600,000 vs. >600,000 IU/ml), ALT levels (≤105 vs. >105 U/ml) and treatment modality (PEG-INF vs. standard IFN). None of these variables turned out to be significant in our study. While younger patients have shown better treatment response in large multicentric studies (87,89), the association with female gender has been inconsistent (9,67,87,89). A lower body weight has been found to be predictive of SVR (87,89). Diabetes mellitus has emerged as a cofactor in failure to achieve SVR (101). Lower baseline viral loads have been associated with greater response rates (83,86). We were not able to establish an association of treatment response with any of these factors. A previous study from our centre (124) also attempted to study the effect of age, gender and pre-treatment viral load on SVR, but failed to find any association. Similarly Gupta *et al.* (18) did not find age, gender, body mass index and base line viral load to influence SVR.

Diabetes mellitus has been found to be a predictor of non response to interferon based treatment in patients with chronic hepatitis C. In a case control study (101), 61 diabetic and 122 non-diabetic chronic HCV patients were treated with standard regimen of PEG-INF /RBV as per the infecting viral genotype. The diabetic patients were older, had significantly high body mass index, greater steatosis and advanced hepatic fibrosis, and showed a significantly lower SVR rates (23%) compared to the compared to non-diabetic HCV patients (46%). It is hypothesised that hyperinsulinemia associated with type 2 diabetes accelerates progression of liver fibrosis. Of the 34 patients analyzed in our study, 9 were diabetics. There was no difference in the response rate in diabetics and non diabetics.

The association between baseline ALT levels and viral response in HCV infection is not as distinct as in chronic hepatitis B infection (97). While Fried *et al*.(87) could not find an association of liver enzymes with SVR, Shiffman *et al*.(89) observed such a correlation in HCV genotype 2 and 3 infected patients. In our study, 21 out of 34 patients had pre-treatment ALT levels greater than 105 U/L, of whom 16 achieved SVR (76%). Of the 8 patients who had ALT levels ≤ 105 U/L, 3 achieved SVR (37.5%). The data was not available for 5 patients, as they had been treated at some other centre and had been referred to us later. Though the difference between the two groups was not statistically significant, a trend was observed (p=0.08). In a previous report from our centre (124), baseline ALT levels were not found to be associated with treatment response. Similarly, Gupta *et al*.(18) did not such an association, though their cut off was 100U/L.

Modification of standard interferon by attachment of a polyethylene glycol moiety has been reported to improve virological response rates by over 10% due the improvement of the pharmacokinetic profile (74,84) . Of the 34 patients we followed up, 21 were on PEG-INF and RBV combination therapy while the remaining 13 patients were on treatment with a combination of standard IFN and RBV. Of the 21 patients, 10 achieved SVR while 9 of the 13 receiving standard interferon achieved SVR. No difference was found between the two groups in terms of SVR rates. This is in keeping with our previous experience, when David *et al*.(124) in their retrospective analysis of HCV genotype 3 infected patients did not find any difference in the response rates between individuals treated with standard IFN vs. PEG-INF. Studies with greater sample sizes are needed to bring out the picture more clearly.

Another variable analyzed for effect on SVR was RVR. We found RVR to be a strong predictor of SVR. In our analysis of the 34 patients who were followed upto 6 months after cessation of therapy, RVR rates were found to be 71%. RVR was achieved in 100% of the patients who were treatment responders, in sharp contrast to only 20% in patients who turned

out to be treatment non-responders. RVR is considered one of the strongest predictors of sustained viral clearance. In a large randomized controlled trial by Ferenci and colleagues involving (136) 1121 patients, RVR was found to be highly predictive of long term response in patients treated with PEG-INF plus placebo, PEG-INF plus RBV and standard IFN plus RBV. Gupta *et al.* (18) and Firdaus *et al.* (19) also replicated the finding that RVR was strongly associated with response to interferon based therapy for chronic hepatitis C. While IL28B genotype is identified as the strongest *pre-treatment* predictor of sustained viral clearance, RVR is known to be the key *on-treatment* response predictor(132).

Advanced liver fibrosis and cirrhosis are major predictors of non-response to treatment, across all viral genotypes. The impact of these SNPs on progression of the liver disease is not clearly established, though an association has been seen (19). Our study did not address the histopathological features seen on liver biopsy, and their association with treatment response. Liver biopsy, being an invasive procedure, was not performed on any of the 34 patients we analyzed. If available, liver biopsy samples could provide not only the histopathological picture, but would also help in determining the expression levels of intahepatic ISGs, which would predict the response to interferon based therapy, as discussed above.

We attempted a multivariate analysis of the factors impacting response but, we did not find any independent factors associated with sustained viral response. This is largely attributable to the limited numbers studied, which was primarily due to the time frame for completion of this study and absence of regular follow up in many patients due to the long distances patients have to travel to come for review to our tertiary care centre. A larger study will be required to analyze the effect of these host and viral factors on treatment response.

CONCLUSION

In this study, we determined the frequency and distribution of IL28B polymorphisms at loci rs12979860 and rs8099917 in 57 HCVinfected patients. SVR rates in genotype 1 and 3 were analyzed and compared. We studied the impact of IL28B single nucleotide polymorphisms on response to treatment in chronic hepatitis genotype 1 and 3 infections. We also studied the association of other factors like age, gender, body mass index, diabetes, rapid viral response, pre-treatment viral load, baseline ALT levels and treatment modality (pegylated vs. standard interferon) with sustained virological response.

The CC allele at rs12979860 and TT at rs8099917 were found in majority of the cases (60% and 72% respectively). We found similar response rates in HCV genotype 1 and 3 infections (57% and 54% respectively).The CC genotype at rs12979860 loci was found to be associated with sustained viral response. No association was found between rs8099917 polymorphism and treatment response. Rapid viral response was found to be most predictive of sustained viral clearance. An association could not be found with younger age, female gender, lower body mass index, non-diabetic state, lower pre-treatment ALT levels, lower baseline viral loads and sustained response to anti-viral therapy. The response rate seen with PEG-IFN was the same as that with standard interferon therapy. The two polymorphisms were not found to be associated with EVR and end of treatment response. However an association of CC genotype at rs12979860 with RVRwas seen.

Ours is the first attempt from the country to study the impact of IL28B polymorphisms on HCV genotype 1 infected patients in addition to genotype 3. We intend to continue the study on larger number of samples to validate our findings and to bring out the associations more strongly.

Bibliography:

1. Lavanchy D. Evolving epidemiology of hepatitis C virus. Clin Microbiol Infect. 2011;17(2):107–15.

2. Mohd Hanafiah K, Groeger J, Flaxman AD, Wiersma ST. Global epidemiology of hepatitis C virus infection: new estimates of age-specific antibody to HCV seroprevalence. Hepatol Baltim Md. 2013 Apr;57(4):1333–42.

3. Abraham P. Viral hepatitis in India. Clin Lab Med. 2012 Jun;32(2):159–74.

4. Mukhopadhyaya A. Hepatitis C in India. J Biosci. 2008 Nov;33(4):465–73.

5. Missiha SB, Ostrowski M, Heathcote EJ. Disease progression in chronic hepatitis C: modifiable and nonmodifiable factors. Gastroenterology. 2008 May;134(6):1699–714.

6. Smith DB, Bukh J, Kuiken C, Muerhoff AS, Rice CM, Stapleton JT, et al. Expanded classification of hepatitis C virus into 7 genotypes and 67 subtypes: updated criteria and genotype assignment web resource. Hepatol Baltim Md. 2014 Jan;59(1):318–27.

7. Christdas J, Sivakumar J, David J, Daniel HDJ, Raghuraman S, Abraham P. Genotypes of hepatitis C virus in the Indian sub-continent: a decade-long experience from a tertiary care hospital in South India. Indian J Med Microbiol. 2013 Dec;31(4):349–53.

8. Ghany MG, Nelson DR, Strader DB, Thomas DL, Seeff LB. An update on treatment of genotype 1 chronic hepatitis C virus infection: 2011 practice guideline by the American Association for the Study of Liver Diseases. Hepatol Baltim Md. 2011 Oct;54(4):1433–44.

9. Feld JJ, Hoofnagle JH. Mechanism of action of interferon and ribavirin in treatment of hepatitis C. Nature. 2005 Aug 18;436(7053):967–72.

10. Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature. 2009 Sep 17;461(7262):399–401.

11. Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, et al. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet. 2009 Oct;41(10):1105–9.

12. Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, et al. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet. 2009 Oct;41(10):1100–4.

13. Bellanti F, Vendemiale G, Altomare E, Serviddio G. The Impact of Interferon Lambda 3 Gene Polymorphism on Natural Course and Treatment of Hepatitis C. Clin Dev Immunol. 2012;2012:1–9.

14. Thomas DL, Thio CL, Martin MP, Qi Y, Ge D, O'hUigin C, et al. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. Nature. 2009 Oct 8;461(7265):798–801.

15. Asselah T, De Muynck S, Broët P, Masliah-Planchon J, Blanluet M, Bièche I, et al. IL28B polymorphism is associated with treatment response in patients with genotype 4 chronic hepatitis C. J Hepatol. 2012 Mar;56(3):527–32.

16. Jimenez-Sousa MA, Fernandez-Rodriguez A, Guzman-Fulgencio M, Garcia-Alvarez M, Resino S. Meta-analysis: implications of interleukin-28B polymorphisms in spontaneous and treatment-related clearance for patients with hepatitis C. BMC Med. 2013 Jan 8;11:6.

17. Sivaprasad S, Rao PN, Gupta R, Ashwini K, Reddy DN. The Distribution of Genotype and Allelic Frequency of IL28B Gene Polymorphism in Andhra Pradesh, India. J Clin Exp Hepatol. 2012 Jun;2(2):112–5.

18. Gupta AC, Trehanpati N, Sukriti S, Hissar S, Midha V, Sood A, et al. Interleukin-28b CC genotype predicts early treatment response and CT/TT genotypes predicts non-response in patients infected with HCV genotype 3. J Med Virol. 2014 Apr;86(4):707–12.

19. Firdaus R, Biswas A, Saha K, Mukherjee A, Chaudhuri S, Chandra A, et al. Impact of Host IL28B rs12979860, rs8099917 in Interferon Responsiveness and Advanced Liver Disease in Chronic Genotype 3 Hepatitis C Patients. PLoS ONE. 2014 Jun 10;9(6):e99126.

20. Averhoff FM, Glass N, Holtzman D. Global burden of hepatitis C: considerations for healthcare providers in the United States. Clin Infect Dis Off Publ Infect Dis Soc Am. 2012 Jul;55 Suppl 1:S10–5.

21. Chowdhury A, Santra A, Chaudhuri S, Dhali GK, Chaudhuri S, Maity SG, et al. Hepatitis C virus infection in the general population: a community-based study in West Bengal, India. Hepatol Baltim Md. 2003 Apr;37(4):802–9.

22. Chadha MS, Tungatkar SP, Arankalle VA. Insignificant prevalence of antibodies to hepatitis C in a rural area of western Maharashtra. Indian J Gastroenterol Off J Indian Soc Gastroenterol. 1999 Mar;18(1):22–3.

23. Chandra M, Khaja MN, Farees N, Poduri CD, Hussain MM, Aejaz Habeeb M, et al. Prevalence, risk factors and genotype distribution of HCV and HBV infection in the tribal population: a community based study in south India. Trop Gastroenterol Off J Dig Dis Found. 2003 Dec;24(4):193–5.

24. Khaja MN, Madhavi C, Thippavazzula R, Nafeesa F, Habib AM, Habibullah CM, et al. High prevalence of hepatitis C virus infection and genotype distribution among general population, blood donors and risk groups. Infect Genet Evol J Mol Epidemiol Evol Genet Infect Dis. 2006 May;6(3):198–204.

25. Acharya SK, Madan K, Dattagupta S, Panda SK. Viral hepatitis in India. Natl Med J India. 2006 Aug;19(4):203–17.

26. Nandi J, Bhawalkar V, Mody H, Elavia A, Desai PK, Banerjee K. Detection of HIV-1, HBV and HCV antibodies in blood donors from Surat, western India. Vox Sang. 1994;67(4):406–7.

27. Jha J, Banerjee K, Arankalle VA. A high prevalence of antibodies to hepatitis C virus among commercial plasma donors from Western India. J Viral Hepat. 1995;2(5):257–60.

28. Choo QL, Richman KH, Han JH, Berger K, Lee C, Dong C, et al. Genetic organization and diversity of the hepatitis C virus. Proc Natl Acad Sci U S A. 1991 Mar 15;88(6):2451–5.

29. Bukh J, Purcell RH, Miller RH. Sequence analysis of the 5' noncoding region of hepatitis C virus. Proc Natl Acad Sci U S A. 1992 Jun 1;89(11):4942–6.

30. Friebe P, Lohmann V, Krieger N, Bartenschlager R. Sequences in the 5' nontranslated region of hepatitis C virus required for RNA replication. J Virol. 2001 Dec;75(24):12047–57.

31. Friebe P, Bartenschlager R. Genetic analysis of sequences in the 3' nontranslated region of hepatitis C virus that are important for RNA replication. J Virol. 2002 Jun;76(11):5326–38.

32. You S, Rice CM. 3' RNA elements in hepatitis C virus replication: kissing partners and long poly(U). J Virol. 2008 Jan;82(1):184–95.

33. Klein KC, Dellos SR, Lingappa JR. Identification of residues in the hepatitis C virus core protein that are critical for capsid assembly in a cell-free system. J Virol. 2005 Jun;79(11):6814–26.

34. Bartosch B, Dubuisson J, Cosset F-L. Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complexes. J Exp Med. 2003 Mar 3;197(5):633–42.

35. Simmonds P. Genetic diversity and evolution of hepatitis C virus--15 years on. J Gen Virol. 2004 Nov;85(Pt 11):3173–88.

36. Griffin SDC, Beales LP, Clarke DS, Worsfold O, Evans SD, Jaeger J, et al. The p7 protein of hepatitis C virus forms an ion channel that is blocked by the antiviral drug, Amantadine. FEBS Lett. 2003 Jan 30;535(1-3):34–8.

37. Jirasko V, Montserret R, Appel N, Janvier A, Eustachi L, Brohm C, et al. Structural and functional characterization of nonstructural protein 2 for its role in hepatitis C virus assembly. J Biol Chem. 2008 Oct 17;283(42):28546–62.

38. Lindenbach BD, Rice CM. Unravelling hepatitis C virus replication from genome to function. Nature. 2005 Aug 18;436(7053):933–8.

39. Martell M, Esteban JI, Quer J, Genescà J, Weiner A, Esteban R, et al. Hepatitis C virus (HCV) circulates as a population of different but closely related genomes: quasispecies nature of HCV genome distribution. J Virol. 1992 May;66(5):3225–9.

40. Robinson M, Tian Y, Delaney WE, Greenstein AE. Preexisting drug-resistance mutations reveal unique barriers to resistance for distinct antivirals. Proc Natl Acad Sci U S A. 2011 Jun 21;108(25):10290–5.

41. Simmonds P, Bukh J, Combet C, Deléage G, Enomoto N, Feinstone S, et al. Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. Hepatol Baltim Md. 2005 Oct;42(4):962–73.

42. Zein NN. Clinical significance of hepatitis C virus genotypes. Clin Microbiol Rev. 2000 Apr;13(2):223–35.

43. Simmonds P, Holmes EC, Cha TA, Chan SW, McOmish F, Irvine B, et al. Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region. J Gen Virol. 1993 Nov;74 (Pt 11):2391–9.

44. Raghuraman S, Abraham P, Sridharan G, Daniel HD, Ramakrishna BS, Shaji RV. HCV genotype 4--an emerging threat as a cause of chronic liver disease in Indian (south) patients. J Clin Virol 2004 Dec;31(4):253–8.

45. Raghuraman S, Abraham P, Sridharan G, Ramakrishna BS. Hepatitis C virus genotype 6 infection in India. Indian J Gastroenterol Off J Indian Soc Gastroenterol. 2005 Apr;24(2):72–3.

46. Hissar SS, Goyal A, Kumar M, Pandey C, Suneetha PV, Sood A, et al. Hepatitis C virus genotype 3 predominates in North and Central India and is associated with significant histopathologic liver disease. J Med Virol. 2006 Apr;78(4):452–8.

47. Chakravarti A, Ashraf A, Malik S. A study of changing trends of prevalence and genotypic distribution of hepatitis C virus among high risk groups in North India. Indian J Med Microbiol. 2013 Dec;31(4):354–9.

48. Chakravarti A, Dogra G, Verma V, Srivastava AP. Distribution pattern of HCV genotypes & its association with viral load. Indian J Med Res. 2011 Mar;133:326–31.

49. Chaudhuri S, Das S, Chowdhury A, Santra A, Bhattacharya SK, Naik TN. Molecular epidemiology of HCV infection among acute and chronic liver disease patients in Kolkata, India. J Clin Virol Off Publ Pan Am Soc Clin Virol. 2005 Jan;32(1):38–46.

50. Singh S, Malhotra V, Sarin SK. Distribution of hepatitis C virus genotypes in patients with chronic hepatitis C infection in India. Indian J Med Res. 2004 Apr;119(4):145–8.

51. Raghuraman S, Shaji RV, Sridharan G, Radhakrishnan S, Chandy G, Ramakrishna BS, et al. Distribution of the different genotypes of HCV among patients attending a tertiary care hospital in south India. J Clin Virol 2003 Jan;26(1):61–9.

52. Das BR, Kundu B, Khandapkar R, Sahni S. Geographical distribution of hepatitis C virus genotypes in India. Indian J Pathol Microbiol. 2002 Jul;45(3):323–8.

53. Amarapurkar D, Dhorda M, Kirpalani A, Amarapurkar A, Kankonkar S. Prevalence of hepatitis C genotypes in Indian patients and their clinical significance. J Assoc Physicians India. 2001 Oct;49:983–5.

54. Valliammai T, Thyagarajan SP, Zuckerman AJ, Harrison TJ. Diversity of genotypes of hepatitis C virus in southern India. J Gen Virol. 1995 Mar;76 (Pt 3):711–6.

55. Hoofnagle JH. Course and outcome of hepatitis C. Hepatol Baltim Md. 2002 Nov;36(5 Suppl 1):S21–9.

56. Fattovich G, Stroffolini T, Zagni I, Donato F. Hepatocellular carcinoma in cirrhosis: incidence and risk factors. Gastroenterology. 2004 Nov;127(5 Suppl 1):S35–50.

57. Gale M, Foy EM. Evasion of intracellular host defence by hepatitis C virus. Nature. 2005 Aug 18;436(7053):939–45.

58. Kawai T, Akira S. Toll-like receptor and RIG-I-like receptor signaling. Ann N Y Acad Sci. 2008 Nov;1143:1–20.

59. Rehermann B. Hepatitis C virus versus innate and adaptive immune responses: a tale of coevolution and coexistence. J Clin Invest. 2009 Jul;119(7):1745–54.

60. Bowen DG, Walker CM. Adaptive immune responses in acute and chronic hepatitis C virus infection. Nature. 2005 Aug 18;436(7053):946–52.

61. Edwards VC, Tarr AW, Urbanowicz RA, Ball JK. The role of neutralizing antibodies in hepatitis C virus infection. J Gen Virol. 2012 Jan;93(Pt 1):1–19.

62. Urbani S, Amadei B, Fisicaro P, Tola D, Orlandini A, Sacchelli L, et al. Outcome of acute hepatitis C is related to virus-specific CD4 function and maturation of antiviral memory CD8 responses. Hepatol Baltim Md. 2006 Jul;44(1):126–39.

63. Lechner F, Wong DK, Dunbar PR, Chapman R, Chung RT, Dohrenwend P, et al. Analysis of successful immune responses in persons infected with hepatitis C virus. J Exp Med. 2000 May 1;191(9):1499–512.

64. Ghany MG, Strader DB, Thomas DL, Seeff LB. Diagnosis, management, and treatment of hepatitis C: An update. Hepatology. 2009 Apr 1;49(4):1335–74.

65. Ng V, Saab S. Effects of a sustained virologic response on outcomes of patients with chronic hepatitis C. Clin Gastroenterol Hepatol Off Clin Pract J Am Gastroenterol Assoc. 2011 Nov;9(11):923–30.

66. WHO | Guidelines for the screening, care and treatment of persons with hepatitis C infection [Internet]. WHO. [cited 2014 Aug 20]. Available from: http://www.who.int/hiv/pub/hepatitis/hepatitis-c-guidelines/en/

67. Heim MH. 25 years of interferon-based treatment of chronic hepatitis C: an epoch coming to an end. Nat Rev Immunol. 2013 Jul;13(7):535–42.

68. Dugum M, O'Shea R. Hepatitis C virus: here comes all-oral treatment. Cleve Clin J Med. 2014 Mar;81(3):159–72.

69. Hoofnagle JH, Mullen KD, Jones DB, Rustgi V, Di Bisceglie A, Peters M, et al. Treatment of chronic non-A,non-B hepatitis with recombinant human alpha interferon. A preliminary report. N Engl J Med. 1986 Dec 18;315(25):1575–8.

70. Lau DT, Kleiner DE, Ghany MG, Park Y, Schmid P, Hoofnagle JH. 10-Year follow-up after interferon-alpha therapy for chronic hepatitis C. Hepatol Baltim Md. 1998 Oct;28(4):1121–7.

71. Poynard T, Bedossa P, Chevallier M, Mathurin P, Lemonnier C, Trepo C, et al. A comparison of three interferon alfa-2b regimens for the long-term treatment of chronic non-A, non-B hepatitis. Multicenter Study Group. N Engl J Med. 1995 Jun 1;332(22):1457–62.

72. McHutchison JG, Poynard T. Combination therapy with interferon plus ribavirin for the initial treatment of chronic hepatitis C. Semin Liver Dis. 1999;19 Suppl 1:57–65.

73. McHutchison JG, Gordon SC, Schiff ER, Shiffman ML, Lee WM, Rustgi VK, et al. Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. N Engl J Med. 1998 Nov 19;339(21):1485–92.

74. Zeuzem S, Feinman SV, Rasenack J, Heathcote EJ, Lai MY, Gane E, et al. Peginterferon alfa-2a in patients with chronic hepatitis C. N Engl J Med. 2000 Dec 7;343(23):1666–72.

75. Bekisz J, Schmeisser H, Hernandez J, Goldman ND, Zoon KC. Human interferons alpha, beta and omega. Growth Factors Chur Switz. 2004 Dec;22(4):243–51.

76. Frese M, Schwärzle V, Barth K, Krieger N, Lohmann V, Mihm S, et al. Interferon-gamma inhibits replication of subgenomic and genomic hepatitis C virus RNAs. Hepatol Baltim Md. 2002 Mar;35(3):694–703.

77. Thimme R, Binder M, Bartenschlager R. Failure of innate and adaptive immune responses in controlling hepatitis C virus infection. FEMS Microbiol Rev. 2012 May 1;36(3):663–83.

78. Marcellin P, Boyer N, Gervais A, Martinot M, Pouteau M, Castelnau C, et al. Long-term histologic improvement and loss of detectable intrahepatic HCV RNA in patients with chronic hepatitis C and sustained response to interferon-alpha therapy. Ann Intern Med. 1997 Nov 15;127(10):875–81.

79. Lau DT, Kleiner DE, Ghany MG, Park Y, Schmid P, Hoofnagle JH. 10-Year follow-up after interferon-alpha therapy for chronic hepatitis C. Hepatol Baltim Md. 1998 Oct;28(4):1121–7.

80. Shiffman ML. Retreatment of patients with chronic hepatitis C. Hepatol Baltim Md. 2002 Nov;36(5 Suppl 1):S128–34.

81. Fried MW. Side effects of therapy of hepatitis C and their management. Hepatol Baltim Md. 2002 Nov;36(5 Suppl 1):S237–44.

82. Manns MP, Wedemeyer H, Cornberg M. Treating viral hepatitis C: efficacy, side effects, and complications. Gut. 2006 Sep;55(9):1350–9.

83. Zeuzem S. Heterogeneous virologic response rates to interferon-based therapy in patients with chronic hepatitis C: who responds less well? Ann Intern Med. 2004 Mar 2;140(5):370–81.

84. Hadziyannis SJ, Sette H, Morgan TR, Balan V, Diago M, Marcellin P, et al. Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. Ann Intern Med. 2004 Mar 2;140(5):346–55.

85. Jacobson IM, Brown RS, Freilich B, Afdhal N, Kwo PY, Santoro J, et al. Peginterferon alfa-2b and weight-based or flat-dose ribavirin in chronic hepatitis C patients: a randomized trial. Hepatol Baltim Md. 2007 Oct;46(4):971–81.

86. Conjeevaram HS, Fried MW, Jeffers LJ, Terrault NA, Wiley-Lucas TE, Afdhal N, et al. Peginterferon and ribavirin treatment in African American and Caucasian American patients with hepatitis C genotype 1. Gastroenterology. 2006 Aug;131(2):470–7.

87. Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Gonçales FL, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med. 2002 Sep 26;347(13):975–82.

88. Zeuzem S, Hultcrantz R, Bourliere M, Goeser T, Marcellin P, Sanchez-Tapias J, et al. Peginterferon alfa-2b plus ribavirin for treatment of chronic hepatitis C in previously untreated patients infected with HCV genotypes 2 or 3. J Hepatol. 2004 Jun;40(6):993–9.

89. Shiffman ML, Suter F, Bacon BR, Nelson D, Harley H, Solá R, et al. Peginterferon alfa-2a and ribavirin for 16 or 24 weeks in HCV genotype 2 or 3. N Engl J Med. 2007 Jul 12;357(2):124–34.

90. El-Zayadi A-R, Attia M, Barakat EMF, Badran HM, Hamdy H, El-Tawil A, et al. Response of hepatitis C genotype-4 naïve patients to 24 weeks of Peg-interferon-alpha2b/ribavirin or induction-dose interferon-alpha2b/ribavirin/amantadine: a non-randomized controlled study. Am J Gastroenterol. 2005 Nov;100(11):2447–52.

91. Kamal SM, El Tawil AA, Nakano T, He Q, Rasenack J, Hakam SA, et al. Peginterferon {alpha}-2b and ribavirin therapy in chronic hepatitis C genotype 4: impact of treatment duration and viral kinetics on sustained virological response. Gut. 2005 Jun;54(6):858–66.

92. Antaki N, Hermes A, Hadad M, Ftayeh M, Antaki F, Abdo N, et al. Efficacy of interferon plus ribavirin in the treatment of hepatitis C virus genotype 5. J Viral Hepat. 2008 May;15(5):383–6.

93. Legrand-Abravanel F, Sandres-Sauné K, Barange K, Alric L, Moreau J, Desmorat P, et al. Hepatitis C virus genotype 5: epidemiological characteristics and sensitivity to combination therapy with interferon-alpha plus ribavirin. J Infect Dis. 2004 Apr 15;189(8):1397–400.

94. Papastergiou V, Skorda L, Lisgos P, Stampori M, Ntetskas G, Papakonstantinou L, et al. Hepatitis C virus genotype 5: prospective evaluation of peginterferon/ribavirin treatment efficacy and predictive value of on-treatment virological responses for sustained virological response. J Clin Gastroenterol. 2014 Feb;48(2):160–5.

95. Hui C-K, Yuen M-F, Sablon E, Chan AO-O, Wong BC-Y, Lai C-L. Interferon and ribavirin therapy for chronic hepatitis C virus genotype 6: a comparison with genotype 1. J Infect Dis. 2003 Apr 1;187(7):1071–4.

96. Lam KD, Trinh HN, Do ST, Nguyen TT, Garcia RT, Nguyen T, et al. Randomized controlled trial of pegylated interferon-alfa 2a and ribavirin in treatment-naive chronic hepatitis C genotype 6. Hepatol Baltim Md. 2010 Nov;52(5):1573–80.

97. Kau A, Vermehren J, Sarrazin C. Treatment predictors of a sustained virologic response in hepatitis B and C. J Hepatol. 2008 Oct;49(4):634–51.

98. Salmerón J, Casado J, Rueda PM de, Lafuente V, Diago M, Romero-Gómez M, et al. Quasispecies as predictive factor of rapid, early and sustained virological responses in chronic hepatitis C, genotype 1, treated with peginterferon-ribavirin. J Clin Virol Off Publ Pan Am Soc Clin Virol. 2008 Apr;41(4):264–9.

99. Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature. 2009 Sep 17;461(7262):399–401.

100. Patton HM, Patel K, Behling C, Bylund D, Blatt LM, Vallée M, et al. The impact of steatosis on disease progression and early and sustained treatment response in chronic hepatitis C patients. J Hepatol. 2004 Mar;40(3):484–90.

101. Elgouhari HM, Zein CO, Hanouneh I, Feldstein AE, Zein NN. Diabetes mellitus is associated with impaired response to antiviral therapy in chronic hepatitis C infection. Dig Dis Sci. 2009 Dec;54(12):2699–705.

102. Arase Y, Suzuki F, Suzuki Y, Akuta N, Kobayashi M, Kawamura Y, et al. Sustained virological response reduces incidence of onset of type 2 diabetes in chronic hepatitis C. Hepatol Baltim Md. 2009 Mar;49(3):739–44.

103. Huang J-F, Dai C-Y, Yu M-L, Huang C-F, Huang C-I, Yeh M-L, et al. Pegylated interferon plus ribavirin therapy improves pancreatic β-cell function in chronic hepatitis C patients. Liver Int Off J Int Assoc Study Liver. 2011 Sep;31(8):1155–62.

104. Rauch A, Kutalik Z, Descombes P, Cai T, Di Iulio J, Mueller T, et al. Genetic variation in IL28B is associated with chronic hepatitis C and treatment failure: a genome-wide association study. Gastroenterology. 2010 Apr;138(4):1338–45, 1345.e1–7.

105. Lange CM, Zeuzem S. IL28B single nucleotide polymorphisms in the treatment of hepatitis C. J Hepatol. 2011 Sep;55(3):692–701.

106. Urban TJ, Thompson AJ, Bradrick SS, Fellay J, Schuppan D, Cronin KD, et al. IL28B genotype is associated with differential expression of intrahepatic interferon-stimulated genes in patients with chronic hepatitis C. Hepatol Baltim Md. 2010 Dec;52(6):1888–96.

107. Jia Z, Ding Y, Tian S, Niu J, Jiang J. Test of IL28B Polymorphisms in Chronic Hepatitis C Patients Treated with PegIFN and Ribavirin Depends on HCV Genotypes: Results from a Meta-Analysis. PLoS ONE. 2012 Sep 21;7(9):e45698.

108. Tillmann HL, Thompson AJ, Patel K, Wiese M, Tenckhoff H, Nischalke HD, et al. A Polymorphism Near IL28B Is Associated With Spontaneous Clearance of Acute Hepatitis C Virus and Jaundice. Gastroenterology. 2010 Nov 1;139(5):1586–92.e1.

109. Lin C-Y, Chen J-Y, Lin T-N, Jeng W-J, Huang C-H, Huang C-W, et al. IL28B SNP rs12979860 is a critical predictor for on-treatment and sustained virologic response in patients with hepatitis C virus genotype-1 infection. PloS One. 2011;6(3):e18322.

110. McCarthy JJ, Li JH, Thompson A, Suchindran S, Lao XQ, Patel K, et al. Replicated association between an IL28B gene variant and a sustained response to pegylated interferon and ribavirin. Gastroenterology. 2010 Jun;138(7):2307–14.

111. Thompson AJ, Muir AJ, Sulkowski MS, Ge D, Fellay J, Shianna KV, et al. Interleukin-28B polymorphism improves viral kinetics and is the strongest pretreatment predictor of sustained virologic response in genotype 1 hepatitis C virus. Gastroenterology. 2010 Jul;139(1):120–9.e18.

112. De Nicola S, Aghemo A, Rumi MG, Galmozzi E, Valenti L, Soffredini R, et al. Interleukin 28B polymorphism predicts pegylated interferon plus ribavirin treatment outcome in chronic hepatitis C genotype 4. Hepatol Baltim Md. 2012 Feb;55(2):336–42.

113. Liu T, Sha K, Yang L, Wang Y, Zhang L, Liu X, et al. IL-28B polymorphisms correlated with treatment response in HCV-4 mono-infected patients: a meta-analysis. PloS One. 2014;9(3):e91316.

114. Mangia A, Thompson AJ, Santoro R, Piazzolla V, Tillmann HL, Patel K, et al. An IL28B Polymorphism Determines Treatment Response of Hepatitis C Virus Genotype 2 or 3 Patients Who Do Not Achieve a Rapid Virologic Response. Gastroenterology. 2010 Sep;139(3):821–7.e1.

115. Sarrazin C, Susser S, Doehring A, Lange CM, Müller T, Schlecker C, et al. Importance of IL28B gene polymorphisms in hepatitis C virus genotype 2 and 3 infected patients. J Hepatol. 2011 Mar;54(3):415–21.

116. Moghaddam A, Melum E, Reinton N, Ring-Larsen H, Verbaan H, Bjøro K, et al. IL28B genetic variation and treatment response in patients with hepatitis C virus genotype 3 infection. Hepatol Baltim Md. 2011 Mar;53(3):746–54.

117. Scherzer T-M, Hofer H, Staettermayer AF, Rutter K, Beinhardt S, Steindl-Munda P, et al. Early virologic response and IL28B polymorphisms in patients with chronic hepatitis C genotype 3 treated with peginterferon alfa-2a and ribavirin. J Hepatol. 2011 May;54(5):866–71.

118. Chen Y, Xu H-X, Wang L-J, Liu X-X, Mahato RI, Zhao Y-R. Meta-analysis: IL28B polymorphisms predict sustained viral response in HCV patients treated with pegylated interferon-α and ribavirin. Aliment Pharmacol Ther. 2012 Jul;36(2):91–103.

119. Rangnekar AS, Fontana RJ. IL-28B polymorphisms and the response to antiviral therapy in HCV genotype 2 and 3 varies by ethnicity: a meta-analysis. J Viral Hepat. 2013 Jun;20(6):377–84.

120. Mangia A, Mottola L, Santoro R. Interleukin 28B polymorphisms as predictor of response in hepatitis C virus genotype 2 and 3 infected patients. World J Gastroenterol WJG. 2013 Dec 21;19(47):8924–8.

121. Austin MA, Ordovas JM, Eckfeldt JH, Tracy R, Boerwinkle E, Lalouel JM, et al. Guidelines of the National Heart, Lung, and Blood Institute Working Group on Blood Drawing, Processing, and Storage for Genetic Studies. Am J Epidemiol. 1996 Sep 1;144(5):437–41.

122. Sharafi H, Pouryasin A, Alavian SM, Behnava B, Keshvari M, Mehrnoush L, et al. Development and Validation of a Simple, Rapid and Inexpensive PCR-RFLP Method for Genotyping of Common IL28B Polymorphisms: A Useful Pharmacogenetic Tool for Prediction of Hepatitis C Treatment Response. Hepat Mon. 2012 Mar;12(3):190–5.

123. Mellor J, Holmes EC, Jarvis LM, Yap PL, Simmonds P. Investigation of the pattern of hepatitis C virus sequence diversity in different geographical regions: implications for virus classification. The International HCV Collaborative Study Group. J Gen Virol. 1995 Oct;76 (Pt 10):2493–507.

124. David J, Rajasekar A, Daniel HDD, Ngui SL, Ramakrishna B, Zachariah UG, et al. Infection with hepatitis C virus genotype 3--experience of a tertiary health care centre in south India. Indian J Med Microbiol. 2010 Jun;28(2):155–7.

125. Dk A, Nd P, P R, P K. Do different hepatitis C virus genotypes behave differently? Trop Gastroenterol. 2008 Jun 27;28(3):99–104.

126. Freshwater DA, O'Donnell K, Mutimer DJ. Inferior response of Asian vs non-Asian hepatitis C genotype 3 infection to combination antiviral therapy. J Viral Hepat. 2008 Feb;15(2):115–9.

127. Missiha S, Heathcote J, Arenovich T, Khan K, Canadian Pegasys Expanded Access Group. Impact of asian race on response to combination therapy with peginterferon alfa-2a and ribavirin in chronic hepatitis C. Am J Gastroenterol. 2007 Oct;102(10):2181–8.

128. Yu M-L, Chuang W-L. Treatment of chronic hepatitis C in Asia: when East meets West. J Gastroenterol Hepatol. 2009 Mar;24(3):336–45.

129. Hazari S, Panda SK, Gupta SD, Batra Y, Singh R, Acharya SK. Treatment of hepatitis C virus infection in patients of northern India. J Gastroenterol Hepatol. 2004 Sep;19(9):1058–65.

130. Rao P, Koshy A, Philip J, Premaletha N, Varghese J, Narayanasamy K, et al. Pegylated interferon alfa-2b plus ribavirin for treatment of chronic hepatitis C. World J Hepatol. 2014 Jul 27;6(7):520–6.

131. Honda M, Sakai A, Yamashita T, Nakamoto Y, Mizukoshi E, Sakai Y, et al. Hepatic ISG expression is associated with genetic variation in interleukin 28B and the outcome of IFN therapy for chronic hepatitis C. Gastroenterology. 2010 Aug;139(2):499–509.

132. Clark PJ, Thompson AJ, McHutchison JG. IL28B genomic-based treatment paradigms for patients with chronic hepatitis C infection: the future of personalized HCV therapies. Am J Gastroenterol. 2011 Jan;106(1):38–45.

133. Thompson AJ, McHutchison JG. Will IL28B polymorphism remain relevant in the era of direct-acting antiviral agents for hepatitis C virus? Hepatology. 2012 Jul 1;56(1):373–81.

134. Beinhardt S, Rutter K, Stättermayer AF, Ferenci P. Revisiting the predictors of a sustained virologic response in the era of direct-acting antiviral therapy for hepatitis C virus. Clin Infect Dis Off Publ Infect Dis Soc Am. 2013 Jan;56(1):118–22.

135. Hsu C-S, Hsu S-J, Chen H-C, Tseng T-C, Liu C-H, Niu W-F, et al. Association of IL28B gene variations with mathematical modeling of viral kinetics in chronic hepatitis C patients with IFN plus ribavirin therapy. Proc Natl Acad Sci U S A. 2011 Mar 1;108(9):3719–24.

136. Ferenci P, Fried MW, Shiffman ML, Smith CI, Marinos G, Gonçales FL, et al. Predicting sustained virological responses in chronic hepatitis C patients treated with peginterferon alfa-2a (40 KD)/ribavirin. J Hepatol. 2005 Sep;43(3):425–33.

PATIENT INFORMATION SHEET

Study title

Study of the association of IL-28B polymorphisms with virological markers and treatment response in patients with hepatitis C virus infection

Purpose of the study:

Hepatitis C is a virus which causes infection of the liver, which can often progress to chronic liver disease, sometimes resulting in liver failure and liver cancer. It is treated with drugs called interferons and ribavirin, which are expensive and also have some significant side effects. However, chronic nature of the infection makes treatment important.

The severity of the disease and the response to treatment depends on a number of viral and human factors. One such human factor is a gene called IL 28B. There are different genotypes the IL 28B gene which predict the response to treatment.

In this study we aim to identify the genotype of IL 28B gene present in patients with hepatitis C virus infection, and correlate it with treatment response.

Description of the procedure:

Relevant patient details will be recorded. Blood sample collected in the department of Clinical Virology as a part of the routine testing will be used to carry out the additional tests in the laboratory.

Risks or discomfort to the Subject:

As no additional procedures will be performed on the participants, the risks are negligible. The participant will not incur any expense for the extra tests done on the blood sample. The cost of the tests will be borne by the research sponsors.

Benefits of the study:

The information gathered from the study will largely help in the prediction of the treatment response of hepatitis C.

Confidentiality:

Only the investigators of this study will be able to access the patient's medical records and the results of the test. Patients identity will not be revealed in any form or release to third parties or published.

Participation:

The patient's participation in the study entirely voluntary and the patient is free to withdraw from the study at any time, without stating any particular reason. Refusal to participate or withdrawal from the study will not involve any penalty or loss of benefits to which the patient is otherwise entitled

Kindly note:

a. Termination - the patient's participation in the study may be terminated if the patient has illnesses which may interfere with the results of the study

b. No additional costs to the patient from participation in the study

Kindly ask questions and clear doubts before participation as some of the medical terms may not be familiar to you.

Contact person: Dr Pragya Ranjan, PG Registrar, Department of Clinical Microbiology

Phone no:0416-228258

INFORMED CONSENT FORM

(For participation in a research study)

Study Title**: Study of the frequency and distribution of IL 28B polymorphisms in hepatitis C virus infected patients and their association with virological markers and treatment response**

Study Number:

Date of Birth / Age: _____

I_____

son/daughter of _____

(i) I confirm that I have read and understood the information sheet dated _____ for the above study and have had the opportunity to ask questions. [ ]

(ii) I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. [ ]

(iii) I understand that those conducting this study, the Ethics Committee and the regulatory authorities will not need my permission to look at my health records both in respect of the current study and any further research that may be conducted in relation to it, even if I withdraw from the trial. I agree to this access. However, I understand that my identity will not be revealed in any information released to third parties or published. [ ]

(iv) I agree not to restrict the use of any data or results that arise from this study provided such a use is only for scientific purpose(s) [ ]

(v) I agree to take part in the above study. [ ]

Signature (or Thumb impression) of the Subject/Legally Acceptable Representative: _____

Date: _____/_____/_____

Signatory's Name: _____

Signature of the Investigator: _____

Date: _____/_____/_____

Study Investigator's Name: _____

Signature of the Witness: _____

Date: _____/_____/_____

Name of the Witness: _____

Serial	Name	Age	Sex	Place	Height(cm)	Weight(kg)	BMI	Genotype	Diabetes	Hosp No
	RADHA GOVINDA RAY	50	M	BANGLADESH	159	59	23	3	N	467709f
	AKASH OMER	24	M	UTTAR PRADESH	164	53	20	3	N	425232f
	CHANDRA SHEKHAR PRASAD	62	M	JHARKHAND	160	65	25	3	Y	640212d
	VENKATRAMANA RAJU K.	50	M	ANDHRA PRADESH	162	62	24	3	Y	313771f
	MD IQUBAL AHMAD	48	M	JHARKHAND	162	72	27	1	N	251034f
	ASHISH KUMAR BHATTACHARJEE	45	M	BANGLADESH	.	.	.	3	N	428218d
	SIBANI KANUNGO	51	F	BANGLADESH	158	60	24	3	N	494226f
	BASANTA KUMAR GAULI	48	M	NEPAL	.	80	.	1	N	896218d
	MUTHU P.	34	M	TAMIL NADU	168	55	19	3	N	262419f
	MARIA SELVI	56	F	TAMIL NADU	148	59	27	4	N	358998d
	UJJWAL CHOUDHURI	49	M	WEST BENGAL	.	69	.	3	N	544020c
	INDRANI BANERJEE	47	F	WEST BENGAL	152	71	31	3	N	239655f
	SAKUNTHALA SUBRAMANNIAN T.	47	F	KERALA	151	74	32	3	N	662495F
	PARBATI PANDAY	38	M	NEPAL	153	72	31	1	N	187446F
	NASIMA BEGUM	34	F	BANGLADESH	148	56	26	1	N	248092f
	KHURSHIDA BANU	49	F	JHARKHAND	150	53	24	3	N	213471f
	SAHIGUN NISA	56	F	JHARKHAND	.	61	.	3	N	700396d
	MONORAMA BHOWMICK	57	F	WEST BENGAL	143	48	23	1	N	079276f
	RANJIT KARMAKAR	30	M	WEST BENGAL	165	52	19	3	N	151149f
	RATTON AKON	62	M	BANGLADESH	155	71	30	3	N	675725d
	SUREN SEN	48	M	BANGLADESH	.	73	.	3	Y	994356c
	MOAHMMAD KAMAL AKON	59	M	BANGLADESH	170	68	24	3	Y	855095f
	SAHEDA BEGUM	36	F	BANGLADESH	157	72	29	3	N	642680f
	MD. MIZANUR RAHMAN	43	M	BANGLADESH	175	61	20	3	Y	858343f
	SUNDARI S.	50	F	ANDHRA PRADESH	.	52	.	3	N	493675c
	THANGJAM HEMA CHANDRA SINGH	55	M	MANIPUR	160	63	25	3	N	794380f
	JAIPRAKASH SINHA	58	M	JHARKHAND	159	61	24	1	Y	313363F
	RAMU C.	24	M	TAMIL NADU		43	.	3	N	720206D
	RAMAKRISHNAN j	61	M	TAMIL NADU		69	.	3	N	817250d
	MAKHAN CH PAL	56	M	TRIPURA		55	.	1	Y	348893F
	HARIKRISHNAN	41	M	TAMIL NADU		64	.	3	N	764268B
	M K BUNDELA	57	M	JHARKHAND	181	82	25	3	N	608664f
	MOHAMMED PERWAZ AKTHER	44	M	JHARKHAND	171	68	33	3	Y	663264f

Rx details	Rx duration	Rx Starting date	Rx End date	Liver biopsy	AST	ALT
Peg IFN + Rib	6m	May-13	Nov-13	not done	165	271
IFN + Rib	6m	Apr-13	Nov-13	not done	48	61
Peg IFN + Rib	6m	May-12	Oct-12	not done	60	52
Peg IFN + Rib	6m	Nov-12	Apr-13	not done	47	72
IFN + Rib	18m	Oct-12	Mar-14	not done	92	70
Peg IFN + Rib	6m	2008	2008	not done	.	.
IFN + Rib	6m	Jun-13	Dec-13	not done	78	64
IFN + Rib	12m	Apr-11	Apr-12	not done	34	25
IFN + Rib	4m	Sep-13	Dec-13	not done	13	10
Peg IFN + Rib	12m	Jan-09	Dec-09	not done	50	36
Peg IFN + Rib	6m(Previous Relapser)	Sep-07	Feb-08	not done	52	129
Peg IFN + Rib	6m	Jun-13	Dec-13	not done	36	30
IFN + Rib	6m (outside)	Aug-11	Feb-12	not done	.	.
IFN + Rib	12m	May-12	May-13	not done	74	84
IFN + Rib	12m	Aug-12	Aug-13	not done	36	32
IFN + Rib	6m	Aug-12	Feb-13	not done	81	66
Peg IFN + Rib	6m	Oct-10	Mar-11	not done	87	91
Peg IFN + Rib	12m	Aug-12	Jul-13	not done	90	77
Peg IFN + Rib	6m	Apr-12	Sep-12	not done	99	128
Peg IFN + Rib	6m	May-10	Nov-10	not done	51	34
IFN + Rib	6m	Dec-07	May-08	not done	51	86
Peg IFN + Rib	6m(outside)	Nov-13	May-13	not done	.	.
Peg IFN + Rib	6m	Sep-13	Feb-14	not done	79	56
Peg IFN + Rib	6m (outside)	Mar-10	Sep-10	not done	.	.
Peg IFN + Rib	6m	Apr-10	Oct-10	not done	.	64
Peg IFN + Rib	6m (outside)	2013		not done	.	.
Peg IFN + Rib	12m	Jan-13	Feb-14	not done	163	208
IFN + Rib	6m	Sep-11	Feb-12	not done	27	26
Peg IFN + Rib	6m	Oct-11	Mar-12	not done	150	79
IFN + Rib	12m	Dec-12	Dec-13	not done	90	117
Peg IFN + Rib	6m in 2009			not done	51	92
Peg IFN + Rib	6m	Aug-13	Feb-14	not done	192	165
IFN + Rib	6m	Sep-13	Feb-14	not done	103	169

Pre Rx Viral Load	RS 60	RS 17	RVR	EVR	Extra	ETR	Extra	SVR	Beyond	
5300000	CC	TT	Y	Y				Y		
710000	CC	TT		Y		Y		Y		
380000	CC	TT		Y		Y		Y	Y	
2500000	CC	TT		Y		Y		Y		
260000	CC	TT	11000	40		N		99000		
.	CT	TT	-	-		-		N	N	
200000	CC	TT	Y					Y		
580000	CT	GT	-	Y		Y		15000		
460000	CT	GT	Y	Y		Y		Y		
1800000	CC	TT	Y	Y		Y		Y	Y	
540000	CT	GT	21000	Y		Y		540000	N	
51000	CT	GT	Y	Y		Y		Y		
79966	CC	TT	-	N		Y		1900	N	
230000	CC	TT	Y	Y		Y		Y		
470000	CC	TT	-	Y		Y			Y(1yr after EOT)	
2800000	CC	TT	Y	Y		Y(7m)			Y(9m after EOT)	
4700000	CC	TT	-	-		Y		-	Y(2yr after EOT)	
7200	CC	TT						Y		
5300000	CT	GT	Y	-		Y		3200000		
32000	CC	TT	-	-		Y		Y	Y	
340000	TT	GG	-	-		Y		530000		
546000	CC	TT	-	-		Y		N	100000	
980000	CT	TT		Y				430000		
7000000	CT	GT	-	-		Y		N	1300000	
2500000	CC	TT	Y	Y		Y		Y	Y	
1480000	CC	TT	-	-		Y		450000		
640000	CC	TT	-	Y		-		300000		
33000	TT	GG	.	.		Y		Y	Y(2.5 yr after EOT)	
180000	CT	GT	26000	Y		Y		210000		
1500000	CC	TT		Y		Y		Y		
210000	CC	TT				Y		Y		
380000	CT	GT	-	Y		Y		21000		
56000	CC	TT	Y			-		Y		

	SHAKH MIRAZUL HUQ	46	M	BANGLADESH	180	85	26	3	Y	344910f
	SUJIT HADA	42	M	NEPAL	164	65		1		409029f
	SARANYA M.	27	F	TAMIL NADU	165	53		4		372052f
	SWAPAN HALDER	57	M	WEST BENGAL	160	48		3		397010f
	SURESH S	45	M	TAMIL NADU	.	64		1		493090f
	REGINAL S.	64	F	TAMIL NADU	154	56		3		359472f
	JANAK KISHORI DEVI	61	F	JHARKHAND	155	55		3		620225f
	ANJANA BISWAS	47	M	WEST BENGAL	.	30		3		443833f
	RAJINI.K	33	F	TAMIL NADU	157	70		3		003827d
	DEVSHANKAR CHOUDHARY	36	M	BIHAR	169	64		3		400457f
	RAKESH KUMAR	33	M	BIHAR	.	54		3		934276d
	RAJ KISHORE PASWAN	29	M	BIHAR	168	66		1		317123f
	KAJARI GHOSH	53	F	WEST BENGAL	153	59		3		449572f
	PRADIP KANTI SHARMA	42	M	BANGLADESH	164	62		3		729877f
	MOHAMMAD NASIR UDDIN	39	M	BANGLADESH	163	46		3		744091f
	MOHAMMED MOHIUDDIN	37	M	BANGLADESH	171	67		3		328789f
	RUKSANA KHATUN	60	F	KARNATAKA	155	58		3		760172f
	SANTHI B	50	F	TAMIL NADU	.	56		3		464879f
	BHARATHI N	47	F	ANDHRA PRADESH	.	63		3		492612f
	ZAFOUR IQBUL	40	M	BANGLADESH	169	78		3		687782f
	MEENA DEVI	41	F	JHARKHAND	151	66		3		589160d
	RUPAK SAHA	46	M	TRIPURA	164	60		3		812434F
	RATNA KUMAR	33	M	TAMIL NADU		75		1		699671F
	SANMUGASUNDARA,	51	M	TAMIL NADU		55	169	1		

Peg IFN + Rib	6M	May-13	Nov-13	not done	104	110
Peg IFN + Rib		May-13		not done	27	22
Peg IFN + Rib	6m	Feb-13	Aug-13	not done	38	52
Peg IFN + Rib		Mar-13	Discontd	not done	178	92
Peg IFN + Rib	12M	Jul-13	Jul-14	not done	41	53
IFN + Rib	6m	Jan-13	Jun-13	not done	113	98
Peg IFN + Rib	6m	Aug-13	Feb-14	not done	62	50
IFN + Rib	6m	Nov-13	May-14	not done	41	57
Peg IFN + Rib	6m	Aug-13	Feb-14	not done	38	39
Peg IFN + Rib	6m	Apr-13	Sep-13	not done	113	91
Peg IFN + Rib	6m	Jan-14	Jun-14		45	58
IFN + Rib		Oct-13			44	77
IFN + Rib	6m	Oct-13	Mar-14		140	122
Peg IFN + Rib	6m	Dec-13	May-14		36	50
Peg IFN + Rib	6m	Dec-13	May-14		39	63
Peg IFN + Rib	6m	Nov-12	Apr-13		42	44
Peg IFN + Rib	6m	Feb-14	Aug-14		93	170
Peg IFN + Rib	continuing	Jun-14			65	44
IFN + Rib	6m	Dec-13	Jun-14		25	28
Peg IFN + Rib	6m	Oct-13	Mar-14		187	181
Peg IFN + Rib	continuing	Jun-14			32	35
Peg IFN + Rib	continuing	Aug-14			114	83
PegINF + Rib	continuing	Aug-14			107	231
IFN + Rib	continuing	Dec-13				

280000	CC	TT	1600	-		Y		50000		
3300000	CT	TT	N	N	6m Yes					
33000	CC	TT	Y	Y	6m Yes					
42000	CC	TT	Y	Y						
43000	TT	GT	Y		6m Yes	Y				
220000	CT	GT	-	Y		N				
4100000	CC	TT	-	N		Y				
12000	TT	GG	-	Y		N				
540000	CC	TT	Y	Y		Y				
800000	CC	TT	-	Y		Y				
450000	CT	TT	-	Y		Y				
440000	CT	GT	N							
1100000	CC	TT	Y	Y		Y				
2000000	CC	TT	Y			Y				
11000000	CT	TT	Y	Y		Y				
250000	CT	TT	Y			Y				
1300000	CC	TT	Y	Y		Y				
140000	CC	TT	Y							
590000	CC	TT	Y	Y						
1400000	CT	GT	N	Y		N	3m N			
4000000	CC	TT	Y							
160000	CT	GT	N							
780000	CT	TT	N							
2500000	CC	TT		Y						