Genetic Heterogeneity, Seroepidemiology and Evolutionary Dynamics of Hepatitis C Virus (HCV) in Association with Antiviral Therapy

Doctor of Philosophy in Molecular Virology

By

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CERTIFICATE

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DEDICATION

I would like to dedicate this thesis to my Parents, who have always been there supporting me for this chapter of my life as without their support and love the road would have been much more difficult to drive on.

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LIST OF ABBREVIATIONS

ALT	Alanine amino transferases
AST	Aspartate amino transferases
ATP	Adenosine nucleotide triphosphate
BDNA	Branched deoxyribonucleic acid probe assay
BLAST	Basic local alignment search tool
BP	Base pairs
CD	Cluster of differentiation
CDC	Centres of Disease Control and Prevention
cDNA	Complementary deoxyribonucleic acid
CI	Confidence interval
CLD	Chronic liver disease
DNA	Deoxyribonucleic acid
E	Envelope protein
E1	Envelop 1 Region
E2	Envelop 2 Region
EDTA	Ethylene diamine tetra acetic acid
EIA	Enzyme immunoassay
ELISA	Enzyme linked immunosorbant assay
EMBL	European molecular biology labs
ER	Endoplasmic reticulum
F1	Portal fibrosis
GTP	Guanosine nucleotide triphosphate
HAART	High activity antiretroviral therapy
HAV	Hepatitis A virus
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HDV	Hepatitis D virus
HEV	Hepatitis E virus
HGV	Hepatitis G virus
HIV	Human immunodeficiency virus
HPVR	Hyper variable region
HSV	Herpes simplex Virus
HSV-2	Herpes simplex Virus-2
HVR	Hypervariable region
IBA	Immunoblot assay
ID	Identity
IDU	Injection drug user
Ig	Immunoglobulin
IgG	Immunoglobulin-G
IFN	Interferon
PegIFN	Pegylated Interferon
IRES	Internal ribosomal entry site
K2EDTA	Di-Potassium Ethylene diamine tetra acetic acid

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PUBLICATIONS

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2. **Kanwal S** and Mahmood T (2012). Hepatitis C Viral Heterogeneity Based on Core Gene and an Attempt to Design Small Interfering RNA Against Strains Resistant to Interferon in Rawalpindi, Pakistan. Hepat. Mon. 12(6):398-407
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1. **Kanwal S** and Mahmood T (2012). Genetic Heterogeneity of HCV in Intrafamilial Transmission among Female Patients. In International Conference on Emerging Infectious Diseases (ICEID in Atlanta, Georgia, USA, 11^{th} – 14^{th} March at the Hyatt Regency Atlanta (Poster).
2. **Kanwal S** and Mahmood T (2012). Association of mutation in HCV core region and response to interferon therapy in patients from Rawalpind, Pakistan. In 22nd Euorpean Congress of Clinical Microbiology and Infectious Diseases, London, United Kingdom, 31^{st} March- 3^{rd} April (Poster).
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ABSTRACT

Hepatitis C virus (HCV) is a global public health problem currently circulating in the human blood for centuries however gets the recognition in 1989. Prevalence and incidence rates vary from country to country. So far, it has affected millions of people from all ethnic origins, all walks of life and all socioeconomic status. Most of the patients develop the persistent infection due to failure in clearance of the virus from their blood and finally leads to cirrhosis and hepato-cellular carcinoma (HCC). Currently.the available conventional antiviral therapy against HCV is interferon (IFN) in combination with Ribavirin (RBV). To attain the sustained virological response (SVR) is the main objective in treating HCV. There are several mechanisms evolved by the HCV that facilitate the persistence of virus and further lead the patient's status as non-responder. Emergence of quasispecies as a result of high genomic variability and immune escape variants is supposed to drive viral resistance to therapy. However, the precise mechanism underlying viral non-response to treatment is still not clear. This provoked us to explore the factors in order to further unravel the mechanism of viral resistance to therapy. This study was basically conducted to explore role of seroepidemiolgy, genetic heterogeneity and evolution in viral resistance to therapy.

In order to accomplish the task, initially 500 HCV ELISA patients were selected who met the selection criteria. Among these, 451 patients were constantly followed throughout the study period and were divided into two groups on the basis of their treatment response. Group 1 constitutes the 376 patients who became HCV RNA negative in response to therapy while group 2 comprised the 75 patients who did not respond to therapy at all and remained HCV RNA positive even till the end of therapy. Patients were diagnosed as non-responders or responders on the basis of viral load determined by Real-Time PCR. From analysis of serum markers both the groups 1 and 2 were compared with positive and negative control groups. Moreover both the treatment groups were followed from the early diagnosis till the 6 months after the end of treatment. Furthermore, to determine the genetic heterogeneity and tracking the evolutionary changes comparative sequence analysis was performed of 75 non-

responders with 75 responders (randomly picked from 376). To proceed further reverse transcriptase PCR was performed by targeting three genomic regions, 5'UTR, core and NS5B. The amplified products were sequenced directly and obtained sequences were cleaned, aligned and submitted to GenBank. Maximum Parsimony (MP) method was used for phylogenetic analysis and dendrograms were dragged to study the evolutionary behavior of different viral types and subtypes of the present study.

All the serum markers related with liver diseases showed significant variation in non-responders as compared to those who become HCV RNA negative in response to therapy. Moreover the patients in group 1 exhibited the presence of genotype 3 which is most prevalent genotype of Pakistan and more susceptible to respond the antiviral therapy whereas the patients who were non-responders possess the HCV isolates with rare genotype in Pakistan i.e 4 and 5 while the most important finding in the present study was the presence of genotype 6q and 6v which is being reported first time from Pakistan. Further, at nucleotide and amino acid level the genetic distance and mutation was high in non-responders in comparison to responders. Difference in percentage composition of individual amino acids was observed between the two groups while amino acid heterogeneity analysis exhibited the different pattern of amino acid substitution and also variation in conserved and variable sites of both the proteins in the two groups. Number of predicted N-phosphorylation and N-glycosylation sites was high in group 2 (non-responders) as compared to group 1 (with SVR). To evaluate the stereo-chemical structure of the proteins, predicted 3D models were selected on the basis of C-score and referred as best for further analysis. According to Ramachandran plot, satisfactory atomic models considered useful for further studies, i.e. to calculate HCV genotypes conservation at structural level and to find out critical binding sites for drug designing. Genetic heterogeneity was found at both nucleotide and amino acid level using different bioinformatics tools. In addition a computational approach was used to design siRNA that could block the synthesis of HCV RNA and can be used against more than one genotypes if become practice in future. Therefore, here it can be assumed that host and viral factors both play their role equally in viral resistance to therapy. According to present results host serum markers i.e. Alanine amino transferase (ALT), Aspartate

amino transferases (AST) , Total Bilirubin (TBL), total protein, albumin, low density lipoprotein (LDL), high density lipoprotein (HDL), total cholesterol (TCOL), triglycerides (TGs), hemoglobin (Hb), prothrombin time (PTT) and platelet count (PLT) act as indicator of viral response to therapy. Moreover, viral heterogeneity both at nucleotide and amino acid level contributed equally in resistance to therapy. Besides this, appearance of rare genotypes is also one of the important factors that hurdle the way to positive response of conventional treatment. Computational analysis showed that genetic diversity, any change or mutation in core region, NS5B and 5'UTR might be the cause of HCV strains to resist IFN therapy. Furthermore, prediction of phosphorylation and glycosylation sites could help in targeting the proper sites for drug designing. A single designed siRNA can be used as an alternative for current therapy against more than one resistant HCV genotypes and can be more economical and affective against HCV if become common practice in future However functional analysis are mandatory to confirm the computational analysis.

INTRODUCTION

Early in 752, Pope Zacharias wrote about "Jaundice of a contagious nature" in a letter to St. Boniface, Bishop of Mainz (Germany) where the effectees were identified. It was described as a highly life threatening disease and accompanied by jaundice affected majority of young people (Kuntz and Kuntz, 2006). Since more than 2500 years infectious jaundice, currently known as hepatitis exhibits the most striking medical illustration in the field of hepatology and has attracted attention of physicians. Globally it had been one of the major sources of epidemics and endemics. Treatments sometimes included silly acts, i.e., live sheep lice were taken orally (Kuntz and Kuntz, 2008). Liver is hepatitis namely hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis B virus related delta agent or hepatitis D virus (HDV) and hepatitis E virus (HEV). Other transfusion transmitted agents belonging to this category of viruses that do not cause hepatitis are hepatitis G virus (HGV) and Transfusion Transmitted Virus (TTV) (Jules and Isselbacher, 2001).

HCV is a vital human pathogen and leads potentially severe complications of affected primarily by systemic infection of acute viral hepatitis. Five different forms of viral agents have been reported to cause acute

persistent HCV infection (Alter, 2007) that encompass hepatocellular carcinoma (HCC), liver cirrhosis and end-stage liver disease. It is the only known member of the genus hepacivirus belong to the family flaviviridae. It can transmit through blood transfusion, organ transplantation, drug injection, dental exposure, body tattoos, and sexual exposure or through vertical transmission.

1.1 HCV VIROLOGY

1.1.1 Structural and Genomic Organization

Size of HCV genome is approximately 9.6 kb. The highly conserved RNA structures exist at 5′ untranslated region (5′ UTR) that contains the internal ribosome entry site (IRES) and at 3′ untranslated region (3′ UTR) that consists of stable stem loop and an internal poly (U)-poly (U/C) tract (Tan, 2006). These UTRs flank a single Open Reading Frame (ORF) encoding a polyprotein of about 3000 amino acids (Penin et al., 2004b and Balvey, 2009) which after processing yield 11 mature proteins (Table 1.1).

Table 1.1: Classification of HCV into structural and nonstructural proteins (www.lan.gov.com).

Division of Protein	Names of Protein	Function	ORF Location	Protein Length (Amino Acid)	MW (kDa)
Structural Protein	Core/P22	ER membrane association, capsid formation, HCV morphogenesis	342-914	191	~23
	E1/gp1	Viral envelop, viral fusion and entry to the host cell	915-1940	192	~33-35
	E2/gp2	Viral envelop, viral fusion and entry to the host cell.	1941-2579	363	~70-72
Non-structural Protein	NS2/p23	ER membrane association, inhibitor of CIDE-B-induced apoptosis, a protease causes self and NS3 cleavage	2769-3419	217	~21-23
	NS3/p70	A serine protease. Causes cleavage of nonstructural proteins downstream to it. Possess RNA helicase activity and interacts with NS5b	3420-5312	631	~69
	NS4a/p8	Cofactor for NS3 protease	5313-5474	54	~6
	NS4b/p27	Integral protein	5475-6257	261	~68
	NS5a/p56-58	Viral replication and regulation of cellular pathways	6258-7601	448	~56-58
	NS5b/p68	RNA-dependent-RNA polymerase	7602-9374	591	~27
Unknown Protein	NS1/P7	Integral membrane protein, $Ca2^{++}$ ion channel (hypothetical function)	2580-2768	63	~7
	Protein F/ ARFP	Viral persistence (hypothetical function)	342-828 (exact location unknown)	161	~16

In the endoplasmic reticulum (ER) of host cell some cellular and viral proteases cleaved a single polypeptide protein and yield mature structural and nonstructural regulatory proteins (Lindenbach and Rice, 2001) (Figure 1.1). Structural proteins encapsulate the viral nucleocapsid and comprise the core (C) and two envelope (E1 and E2) proteins. Six nonstructural (NS) proteins include NS2, NS3, NS4A NS4B, NS5A and NS5B (Reed and Rice, 2000). Structural proteins can be separated from nonstructural protein on the basis of short membrane peptide p7 (which is assumed to be a viroporin) (Table 1.1). Nonstructural proteins NS2 and NS5B are observed to be involved in the processing of polyprotein and replication of virus. Zinc dependant metalloproteinases are specific and distinct proteinase for NS2-NS3 and NS3 which is required for processing of NS protein reaction. N-terminal region of NS3 contain the NS3 proteinase property and cofactor NS4A is utilized for release of residual NS proteins. Phosphorylated protein NS5A is of unknown function while NS5B is RNA dependant RNA polymerase (RdRp) (Penin et al., 2004a). Ribosomal frame shift is suggested to be involved in HCV protein synthesis (Walewski et al., 2002) owing to the presence of four highly conserved structural domains in 5′ UTR and stem loop structure and internal polypyrimidine tract/poly Uridine in 3′ UTR (Penin et al., 2001).

1.1.2 HCV Life Cycle

HCV established the 'ecological niche' in the human liver in consequence of the viral-host complex interactions (Simmonds, 2004). To replicate its genome and get into the cell, the viral particle requires passing the cell membrane (plasma membrane) of a host cell and approaches the nuclear as well as cytosolic components. Entry of HCV into the cell encompasses the multistep but slow process involving the existence of entry factors (Dubuisson et al., 2008). It can also replicate in peripheral blood mononuclear cells (Neumann et al., 1998). The viral particle affix to the host cell by a particular and very strong interaction with CD81 (a B cell surface receptor) receptor on the host cell surface and a viral protein E2 as well as with viral particles when studied *in vitro* (Pileri et al., 1998). Moreover, entry of HCV inside the cell is also possible through binding to low density lipoprotein (LDL) receptors. E1 helps in membrane fusion while E2 work as a Chaperon for E1, which aggregates in misfolded form in the absence of E2 (Michalak et al., 1997 and Flint and McKeating, 1999).

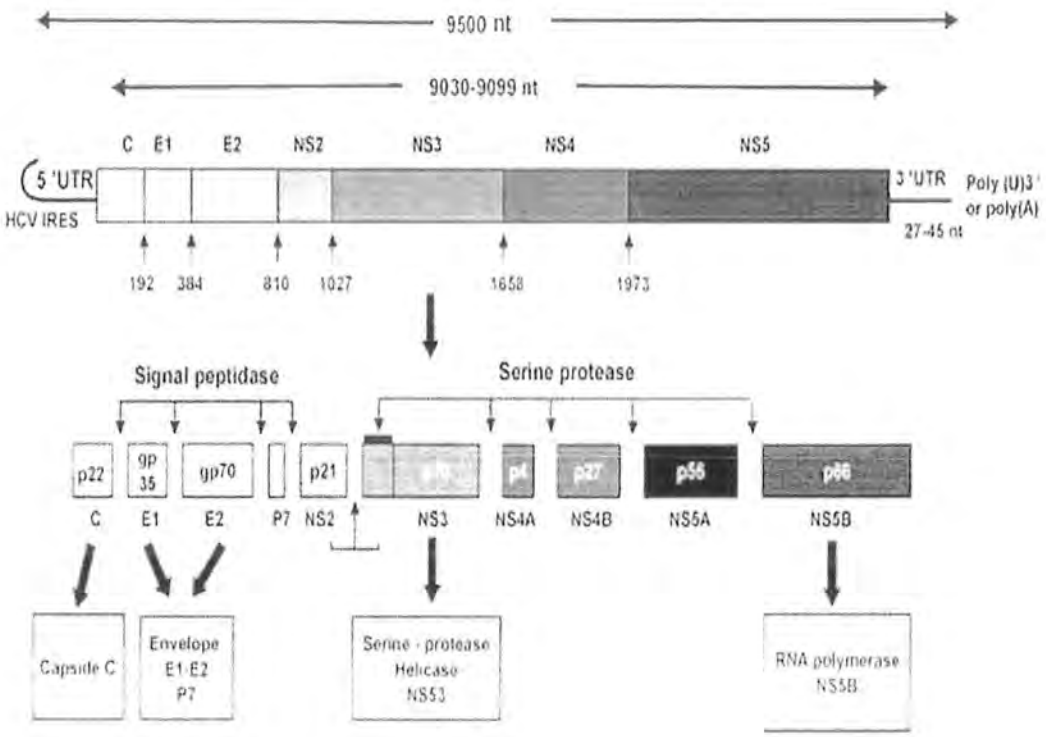

Figure 1.1: HCV genome organization (Giannini and Bréchot, 2003).

Viral binding to a receptor promote receptor-medited endocytosis and leads to the formation of a complex. Opposite to flavivirus translation that is cap-dependent (5′ UTR-m7 Gppp Amp), HCV 5′ UTR is not capped and folds into a complex secondary RNA (Brinton and Dispoto, 1988). The HCV IRES has potential to form pre-initiation complex by binding directly to 40S ribosomal subunit (Spahn et al., 2000 and Otto et al., 2002) (Figure 1.2).

The minus and plus strand of HCV RNA is synthesized by NS5B RdRp and product elongation is achieved by special conformation attain at its 3′ end (Al et al., 1998). RNA primer is synthesized by NS5B by consuming cellular ATP/GTP (Luo et al., 2000) and can copy full length HCV genome *in vitro* (Lohmann et al., 1997). Replication and unwinding of the template through helicase activity is facilitated by NS3 whereas a whole process is regulated by NS5A. Additionally, cellular components poly pyrimidine tract-binding protein (PTB) intermingle with the sequences at the 3′ UTR and Glyceraldehyde-3-phosphate dehydrogenase, binding to the poly (U)-sequence in the 3′ UTR and finally, cellular proteins called p87 and p130 (Ito and Lai 1997; Tsuchihara et al., 1997; Chung and Kaplan, 1999 and Petrik et al., 1999).

Thus, HCV genome is translated into precursor polyprotein, which is cleaved into small proteins by the internal signal sequence and by host signal peptidase. The presence of HCV genome in host ER supports the fact that nucleocapsid achieves its envelope in ER. Presence of N-linked glycans on viral surface supports the exportation of viral particles through golgi via constitutive secretory pathway (Sato et al., 1993).

1.1.3 Genotype Origin

Evolutionary rate of HCV is rapid that result in alarming genetic diversity and leads to the generation of six different genotypes (1–6) which further branched into subtypes, e.g. 1a, 1b, 1c etc (Simmonds, 1999). Several subtypes remain restricted to specific geographical region while others distributed worldwide. These geographically confined subtypes are termed as endemic as they displayed strong restricted genetic heterogeneity, durable localized persistence and low transmission rates (Pybus et al., 2007 and Bostan and Mahmood, 2010). For instance, HCV genotype 3 is endemic all over the South Asia (Mellor et al., 1995) at the same time presence of genotype 6 is also observed in South-East Asia (Pybus et al., 2009).

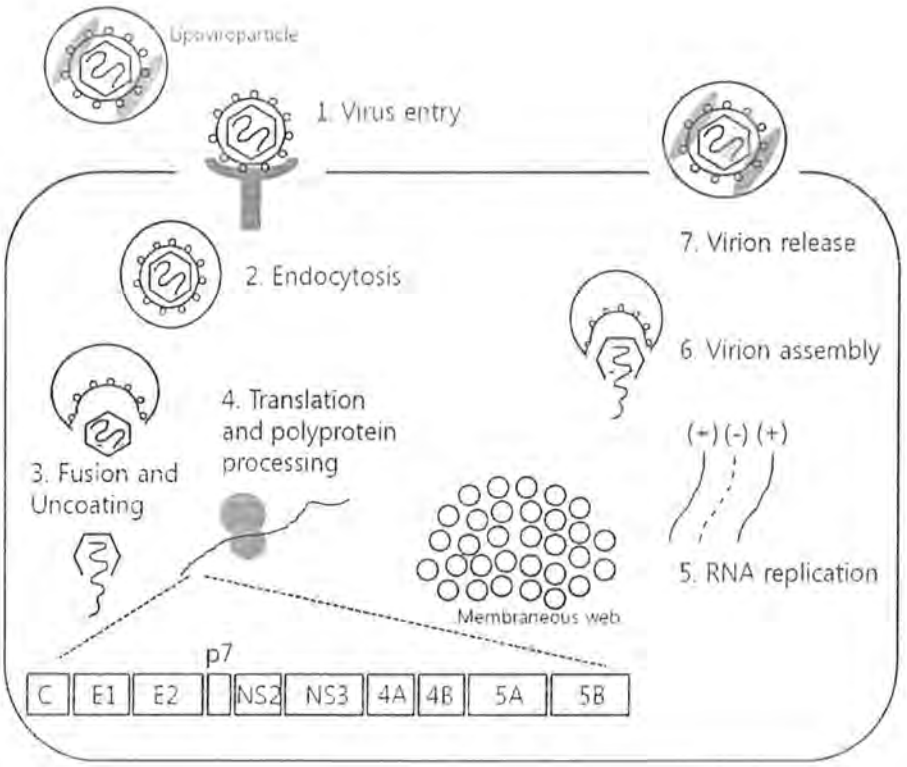

Figure 1.2: Schematic representation of the HCV life cycle (Ploss and Dubuisson, 2012).

In West Africa genotypes 1 and 2 (Ruggieri et al., 1996; Jeannel et al., 1998; Wansbrough-Jones et al., 1998 and Candotti et al., 2003) and in Middle East and Central Africa genotype 4 is reported to be a dominant subtype (Mellor et al., 1995 and Ndjomou et al., 2003). Clinically the determination of genotypes is important for response prediction to, and in formulating the duration of antiviral therapy (Zein et al., 1996). Presently, knowledge about genotype is required prior deciding the treatment strategies for HCV positive patients. This is established by the fact that genotype 1 and 4 resist more to Pegylated interfereon-α (PegIFN-α) and RBV treatment than genotype 2 and 3 (McHutchison et al., 1998 and Zylberberg et al., 2000). Furthermore, it has been reported that chronic HCV patients with genotype 1b infection showed more severe liver disease as compared to patients infected with other genotypes (Silini et al., 1995).

1.2 THE DISEASE

1.2.1 Frequency and Incidence of HCV

HCV is prevalent worldwide and its circulation varies geographically. High prevalence rates have been observed in African and Asian countries while regions with lower occurrence include North America, Europe and Australia (Figure 1.3). A wide range of frequency estimates is available among developing countries as compared to developed countries (Shepard et al., 2005). Lavanchy (2011) described in detail the global prevalence of HCV. According to this review among the developed nations the populous one with low rates of HCV seroprevalence comprise Germany 0.75 %, Canada 1 %, France 1.3 %, and Australia 1.1 %. Relatively higher seroprevalence rates of 1.8 % in USA, 2.4% in Japan and 3.2 % in Italy have been described. Reported HCV prevalence in developing countries include China 2.2 %, India 1.5 %, Indonesia 3.9 %, Pakistan 5.9 % and the highest reported seroprevalence in Egypt is 14 %.

1.2.2 HCV Immunopathogenesis

Macrophages and dendritic cells clear circulating virus and protect host cell from reinfection as they help in transportation of viral proteins to antibody producing B-cells. Helper T-cells phagocytose and breakdown these viral proteins after recognizing them in close vicinity of class II major histocompatibility complex (MHC).

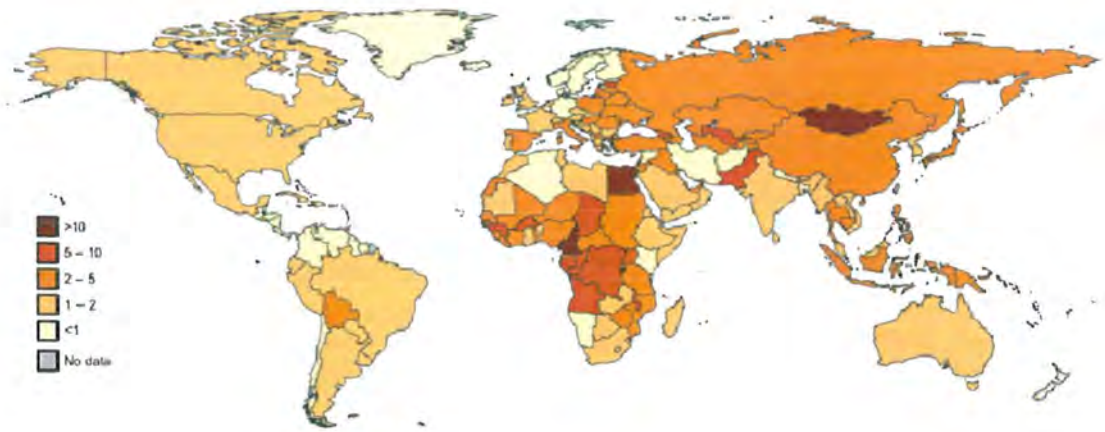

Figure 1.3: Global distribution of HCV (Lavanchy, 2011).

This consequently activates the T-cell receptors for further activation of B-cells followed by initiation and stimulation of virus specific T-cells (Ridge et al., 1998). Immunoregulatory Thi-stimulating cytokines called Th1 and Th2 are involved in mediating these effects. CD8-positive cytotoxic T-cells in the vicinity of class I MHC recognize HCV peptides produced in infected cells and may direct to breakdown of viral infected cells. The liver has been proposed as the major site where activated T-cells are destroyed (Large et al., 1999 and Gale et al., 1999). The evolution of quasispecies and other viral factors have been suggested to contribute to immune evasion by hepatitis C virus. In case of persistent HCV infection, virus-specific cytotoxic T lymphocytes might control viral replication. However, these same effectors may also be responsible for the progressive liver damage which is specific to chronic hepatitis C infection (Freeman et al., 2001).

1.2.3 Seroepidemiology of HCV

AST and ALT are two most important biochemical indicators for heptocellular injury or necrosis. In hepatic disorders their baseline levels can be increased. ALT is more peculiar as it is found mainly in liver cytosol and also in lower concentration elsewhere. It is suggested that yearly screening for liver disease in healthy and asymptomatic patients using ALT and AST levels is not useful (Yano et al., 2001) but their level less than five times upper limit of normal, i.e., upto 250 U/L are common for primary care medicine. HCV is considered as a highly heterogeneous virus and a primary source of chronic liver disease, cirrhosis and HCC. Alberti et al. (2004) reported about 50 % of HCV positive patients with normal ALT levels and two-third patients with mild histological liver lesions. It was commonly observed that short term outcome of initially mild disease is always benign. However, it may lead to the development of liver fibrosis at long term (>5-7 years) follow-up, primarily in patients with elevated and/or variable transaminase levels. Besides this, patients possessing any of genotype of HCV may develop HCC (Mangia et al., 1997). It is also evident that patients with normal ALT and AST levels can have significant liver disease that can develop into chronic hepatocyte injury (e.g., hepatitis C, cirrhosis). In many form of acute and chronic liver injury or steatosis the ratio of AST to ALT is below or equal to 1. This is predominantly found in HCV patients. Though, an AST/ALT ratio more than 2 usually is found in alcoholic hepatitis (Alberti et al., 2004).

Progression of infection occurs under the influence of cofactors like patient's age, alcohol consumption, coinfections with other viral diseases and liver steatosis (fatty infiltration of the liver). Thus, initially mild chronic cases having elevated ALT levels are recommended with suitable antiviral therapy even when contraindications are lacking (Sobesky et al., 2008). Although no correlation was observed for disease severity and duration of infection to genotype distribution, however, for genotype 1b effect of age is strongly related to disease progression (Mangia et al., 1997).

1.3 DIAGNOSIS OF HCV

Generally two types of diagnostic tests can be used to diagnose HCV infection, i.e., serological assays for antibody detection and molecular tests for detection of the viral genome. The detection of anti immunoglobulin G (IgG) in serum using enzyme immuno assay (EIA) is the most important screening test (McHutchinson et al., 1992 and Lauer and Walker, 2001).

1.3.1 Serological Assays

Serological assays include first, second and third generation assays, EIA, enzyme linked immunosorbent assays (ELISA), line probe assay (LiPA), immunoblot assay (IBA), HCV branched DNA-probe assay (bDNA-probe), HCV amplicor monitor assay (amplicor monitor) and HCV core protein assay (core protein) (Kobayashi et al., 1999 and Schroter et al., 2001). The sensitivity of the above mentioned serological assays for the detection of recent infections is good enough, but the drawback of these indirect tests is their inability to differentiate between a recent infection and a past infection, resulting in misclassification of recognized infections (Guy et al., 2009).

1.3.2 Molecular Methods

Limitations of serological assays for HCV antibodies led to the innovation of direct detection of HCV RNA by reverse transcriptase polymerase chain reaction (RT-PCR) (Germer and Zain, 2001). Instability of viral RNA implies careful handling of samples to minimize the chances of false negative results. This is a qualitative test that can detect infection at even less than 100 copies of HCV RNA per milliliter of blood (Beld et al., 2000) and also exhibit efficient results in patients without symptoms (Germer and Zein, 2001 and Lauer and Walker, 2001). Roche Diagnostics introduced Amplicor test for HCV using RT-PCR targeting 5′ UTR (Branchburg, NJ) (Nolte et al., 1995) which was soon followed by the semi automated COBAS

Amplicor HCV test 1.0 (Albadalejo et al., 1998). A more advanced technique employing transcription mediated amplification (TMA) found to be more reliable, robust and sensitive has now become commercially available by Bayer Diagnostics, Emeryville, California (Sarrazin et al., 2000).

1.3.3 Biochemical Markers

Measurement of ALT level is inexpensive, easily available but a non specific test for identification of HCV (NIH 1997a, b). However, it may be best option for monitoring HCV infection and observing the efficacy of therapy but ALT levels do not promise success of antiviral therapy. Physicians still rely on liver biopsy and suggest it the most reliable source for diagnosis (Niederau et al., 1998) and disease progression (Yan et al., 1996). It may also help to identify other causes of liver disease and is therefore, suggested for the preliminary diagnosis chronic HCV infection (NIH 1997a, b and EASL 1999).

1.3.4 Quantitative Detection

Overall, these quantitative assays have been developed not only for the management of infection but also for accurate quantification of virus in serum or plasma even at low levels of viremia (Germer and Zain, 2001). Blood virus level has been shown related to the success of antiviral therapy (McHutchison et al., 1999). Currently three commercial tests are available to quantify HCV infection, i.e., a branched chain DNA assay (Quantiplex HCV RNA, Version 2.0) and two assays involving RT-PCR (COBAS Amplicor HCV Monitor, version 2.0 and HCV Superquant) (Lauer and Walker, 2001). All systems provide reliable but not comparable results (Martinot-Peignoux et al., 2000).

1.3.5 Genotype Determination

Sequence analysis of part of the viral genome after PCR amplification and subsequent phylogenetic analysis is the reference method for HCV genotype determination (Germer et al., 1999). Response of HCV patients to current anti-HCV therapy is also dependant on HCV genotype, with a markedly better response to ribavirin (RBV) and IFN in the genotype 2 or 3 infected population, but concerning the recently introduced protease inhibitors the efficacy in genotype 2 infected patients is modest and absent in patients with genotype 3 (Foster et al., 2011). Therefore genotype determination has a major impact on the clinical management of patients (Hnatyszyn, 2005). A signature sequence identified by reverse hybridization of PCR

to amplify a probe is an alternative method with higher sensitivity to detect viral variants present in lower proportion (Bouchardeau et al., 2007). PCR methods using genotype-specific primers are also available (Okamoto et al., 1992).

1.3.6 Histopathological Assessment

In order to evaluate the natural course of viral hepatitis and to select suitable patients for treatment, it became necessary to standardize the histopathological evaluation. The prevalence and degree of hepatic steatosis and iron deposition as well as the occurrence of bile duct lesions are other important histopathological characteristics.

One of the first classifications of chronic hepatitis introduced difference between chronic active or chronic persistent hepatitis regardless of underlying etiology based on prevalence or absence of pronounced periportal inflammation or "piece-meal necrosis". This classification method was subsequently replaced by more detailed pseudonumerical scoring systems. One such commonly used method was the "histology activity index" (HAI) presented by Knodell et al. (1981). This scoring system was revised in 1995 when an attempt was made to establish consensus among leading pathologists at that time (Ishak et al., 1995). In this "Ishak" system, which is widely accepted, the various aspects of inflammation (interface hepatitis, confluent necrosis, focal lobular inflammation, and portal inflammation) are separately graded on scale from 0 to 4, whereas fibrosis is staged from 0 to 6.

1.4 TREATMENT STRATEGIES

Currently no vaccine or cure against HCV is available because of its highly variable genome. The only known method of prevention is avoiding exposure to the virus. Several therapies have been suggested to minimize the infection. These methods include antiretroviral treatment, inhibitors and small interfering RNA (siRNA).

1.4.1 Interferon (IFNs)

IFNs are naturally occuring cellular proteins exhibit variety of functions such as direct antiviral effect, stimulation of cytokine secretion and recruitment of immune effector cells and initiation of cell differentiation. Several types of IFNs have been approved for human use. In 1980 IFN-α-2a (Davis et al., 1989) was yielded from yeast using DNA recombinant technology (Nagata et al., 1980). Standard monotherapy was the first therapy to cure chronic viral hepatitis introduced in 1980

by Hoofnagle et al. (1986), Davis et al. (1989) and Di Bisceglie et al. (1995). They showed persistent improvement in liver disease and removal of virus in less than 10 % of patients, with the short courses of the therapy. Addition of RBV (oral nucleoside analogue) to the usual regimen was a key advancement. In 1998, Poynard et al and McHutchison et al reported that IFN-α-2b plus RBV when used in combination for 6-12 months resulted in sustained eradication rates of 30-40 %.

Addition of polyethylene glycol (PEG) molecules to conventional IFN led to the modified IFNs known as PegIFNs that exhibit improved persistent incorporation, a slower pace of viral clearance, and a longer half-life as compared to other unmodified. Moreover addition of RBV to PegIFN began a new era in the treatment of HCV (Fried et al., 2002). A recombinant fusion polypeptide of IFN-α-2b and albumin with relatively longer half life known as albIFN (Albuferon) has been reported to exhibit the poor tolerance as compared to PegIFN-α-2a (Nelson et al., 2009). PegIFN-λ is a pegylated type III IFN that binds to a unique receptor with more limited distribution than the type-I IFN receptor. PegIFN-λ is currently investigated in combination with RBV (Shiffman et al., 2007). Another recombinant type 1 IFN is Consensus (C) constituting 166 amino acids. This C was derivative of sequences scanned from numerous natural α IFNs and allocating the most oftenly observed amino acid at each respective position. In an investigation it was suggested that gamma (γ) might act as an effective additive for RBV or IFN-α/RBV therapy specifically in difficult to treat genotype 1/ high viral load patients or patients with less accessibility or conflict for RBV (Kaiser et al., 2005). In general, the new IFNs may progress the ease and accessibility of IFN based therapy.

1.4.2 Nucleoside Analogue

Ribavirin, discovered in 1970, is a guanosine analogue with huge spectrum antiviral activity (Wu et al., 2002). HCV particles become less infectious by treating with RBV. Since the milestone investigations in 1998, RBV has been effectively used in combination with IFN to treat chronic HCV infection (McHutchison et al., 1998 and Poynard et al., 1998).

1.4.3 Specifically Targeted Antiviral Therapy for HCV (STAT-C)

Present researches focused to develop 'Specifically Targeted Antiviral Therapy for HCV' (STAT-C). The proteins of HCV cleaved into smaller proteins with the help of different enzymes. A protease is produced by genomic region NS3 and is further

characterized for its tertiary structure by X-ray crystallography to identify particular promising molecular target sites for future NS3 protease inhibitor therapies. Other potential targets for antiviral therapy in HCV genome constitutes the IRES situated in the 5' UTR of the genome, most potential target for antisense oligonucleotides and ribozymes. RdRp is an additional region in HCV genome for STAT-C. It can be inhibited either by nucleoside, nucleotide analogues or by non-nucleoside analogues (Kapadia et al., 2003 and Lange et al., 2010)

1.4.4 Immune Therapies–Immunomodulatory Agents

Development of new immunomodulatory agents in HCV treatment such as hyperimmune anti-HCV immunoglobulins and therapeutic vaccines (monoclonal antibody against a linear epitope of HCV E2 glycoprotein MBL-HCV1 that neutralizes pseudoviruses from multiple HCV genotypes) have been observed (Dagan et al., 2003 and Puig et al., 2004). However most of them have demonstrated questionable efficacy until now. Currently the development of the agonist of Toll like receptor (TLR) as the most challenging therapeutic agent is going on. A short synthetic oligonucleotides and agonist of TLR, CPG-10101 (Actilon™, COLEY Pharmaceutical Group, USA) is being developed as an antiviral and enhancer drug for the treatment of chronic HCV infection. It attaches to the TLR9 receptors present on human B-cells and plasmacytoid dendritic cells and further induce Th1 immune response like production of tumour necrosis factor, interleukin-12 (IL-12) and IFN-α together with stimulation of B-cell proliferation and antibody production (McHutchison et al., 2006).

1.4.5 Small Interfering RNA (siRNA)

A chemically synthesized double stranded RNA molecule of 20-25 nucleotides are known as small interfering RNA (siRNA), short interfering RNA or silencing RNA plays vital role in medicine. They target the gene that has a 21 nucleotide perfect match. siRNA is involved mostly in RNAi pathway where it knocks down the expression of specific gene by degradation of mRNA.

Usage of siRNA is more useful because it binds directly to specific mRNA and as a result transcription is blocked specifically (Daniel et al., 2006). It possess the potential as molecular therapeutic approach to alternate the current therapy of IFN-α and RBV against HCV which has limited efficiency. HCV RNA is extremely

vulnerable to RNAi-induced suppression, as use of different genes using RNAi in inhibition of HCV RNA levels has been reported (Jahan et al., 2011).

1.5 VIRAL RESPONSE TOWARDS ANTIVIRAL THERAPY

HCV respond to antiviral therapy in three different manners: continuous response termed as sustained virological response (SVR), end-of treatment response and relapse, and non-response. All these patterns possess different implications. Removal of evident HCV RNA from patient's serum and its constant absence even after 6 months of therapy discontinuation is defined as SVR. Investigations on long term follow up patients who achieve SVR revealed that this response is durable in over 95 % of patients (Marcellin et al., 1997 and Lau et al., 1998). Moreover, viral clearance improves the liver histology by reducing the inflammation and degenerating the fibrosis.

Various viral kinetic profiles can be used to characterize the treatment response. Virological responders displayed a quick decline in viral levels followed by second relatively slower phase of decrease till the undetectable levels of circulating virus are achieved (Neumann et al., 1998). Inhibition of viral replication is reflected by the preliminary decrease (phase one), which is calculated as a proportional decline in viral RNA levels within the first 1 to 2 days followed the primary injection of IFN. About 90 to 99 % decrease occurs in the first phase but also varies significantly from 99.9 % (>3 logs) to 0 %. Removal of virus infected cells (by cell death or by suppression of viral replication inside the cell) is considered as the second phase response and is evaluated from the rate of decline in HCV RNA levels subsequent to first phase response. Only a proportion of patients respond in classical viral kinetic manner with thrice-weekly treatment of standard IFN-α and weekly treatment of PegIFN while other patients respond in paradoxical manner (with rebound or multiple phases of response) and some patients have no response at all (Layden and Layden, 2002). Patients in the last category (null or flat response) are almost always non-responders.

1.5.1 HCV Genetic Heterogeneity and Treatment Response

Strong cellular and humoral immune responses induced by HCV infection (Alter et al., 1999) are usually inadequate for viral eradication and prevention of reinfection (Farci et al., 1992). Production of a quasispecies (closely related but genetically distinct variants) permits HCV to avoid the response to host defense and

antiviral therapies. At least 90 % nucleotide sequence homology resulted in two quasispecies (Reddy et al., 2001 and Saito et al., 2007). Therefore, HCV sensitivity to therapy is uneven as this 10 % of genetic divergence can possibly create various viral variants with a diverse receptivity towards treatment. HCV may develop 'evasion variants' from already existing quasispecies pool or by adapting genetic variation. Based on sequencing studies, it has been shown that the resolution of acute HCV infection is associated with an overall reduction in viral quasispecies complexity within the E1 and E2 coding regions of HCV, whereas progression to chronic infection and resistance to IFN therapy is associated with increased viral genetic complexity (Farci et al., 2000 and 2002). HCV evades the host response with the help of a complex combination of processes like signaling interference, effector modulation and continual variation in viral genetics. These evasion strategies are helpful for persistent infection and the spread of HCV (Gale and Foy, 2005).

Production of antibodies inside the host cell after the viral infection stops the reproduction of the virus. Therefore, virus need some change in its genomic region to escape the host immune system (Wesley and Alter, 2000). Besides the other factors the genotype difference play a crucial role in viral response to treatment, as the interaction of certain HCV proteins with intracellular biochemical pathways such as NS5A protein coding region, E2 and intracellular pathways might mediate the effects of IFN (Shoukry et al., 2004). However, adaptive selection leads to viral diversification which further results in viral escape from immune system. HVR-1 in E2 region of virus genome has been reported as dominant neutralization epitope and its carboxy-terminal also possess epitopes for T-helper cells and other cytotoxic response (Aral et al., 2007). In 2011, Li et al. explored that the HCV genome possess significantly higher rate of accumulating synonymous substitutions comparative to non-synonymous substitutions. Moreover, accumulation of deleterious mutations might acquire a threat to viral pathogenesis and persistence. Moreover, a study described that adaptation of HCV to the infected host may be linked to hepatitis C pathogenesis; it suggested that less diversified quasispecies are associated with greater disease severity. Therefore, viral diversity monitoring over time may be an equitable alternative to more-invasive histological monitoring in the clinical setting (Sullivan et al., 2007). Stable region in HCV genome like NS5B may act as target for epidemiological reconstruction. While the HVRs of E2 and NS5A are highly variable

(McGarvey and Houghton, 2005) and may have been formed as a result of specific selection i.e., natural selection for survival of fittest drive virus associated with immune escape such as the HVR-I in E2 may be a target for neutralizing antibody. In this case viral persistence might require continuous mutations to avoid B-cell responses (Farci et al., 2000 and Kantzanou et al., 2003). Differences in viral genetic variability in discrete protein regions have also been linked to differences in response to therapy (Strader et al., 2004).

The high persistence of HCV infections along with the low susceptibility to antiviral treatments, is possibly due to a complex interaction between the of the viral genetic diversity and the host immune response (Weiner et al., 1992; Kato et al., 1993; Taniguchi et al., 1993 and Farci et al., 2000). The selective pressure exerted by the immune system (Chang et al., 1997 and Mondelli et al., 2001) is stronger in those regions with the highest degree of genetic variability (Reed and Rice, 2000). This is the case of HVR1, which has been extensively studied (Okamoto et al., 1992; Ray et al., 1999; Farci et al., 2000; Curran et al., 2002 and Farci et al., 2002) and seems to be involved in target cell recognition and virus attachment (Penin et al., 2001). Moreover, the genetic variability of other regions has also been investigated, for example the interferon-sensitive determining region (ISDR) or the V3 domain of the NS5A protein. Interestingly, a potential role in responsiveness to interferon has been postulated for the ISDR (Gale et al., 1997 and 1998) and the V3 domain (Duverlie et al., 1998 and Durante et al., 2003).

1.5.2 Other Factors Affecting the Viral Response to Therapy

An important determinant of patient response is the concentration of HCV RNA in serum at the time of initiation of antiviral therapy. Constantly higher sustained response rates observed in patients with low baseline HCV RNA levels (usually defined as > 800,000 IU ml^{-1}). The rate of SVR is also dependant on several host factors comprises patient's age, racial background, gender, physiological status i.e obesity and extent of hepatic fibrosis. The most remarkable among these factors is racial difference. For instance response rate of African Americans HCV patients is one-half to one-third those in Caucasians (Muir et al., 2004).

Patients suffering from acute hepatitis C exhibit high rate of response to antiviral therapy (Jaeckel et al., 2001). High persistence rate of virus and evolved chronic infection in 50-80 % of patients characterizes the acute HCV infection. Use of

IFN-γ against acute hepatitis C can lower the chronicity rate to 10 %. Surprisingly, acutely infected patients with hepatitis C (regardless of genotype or initial viral load) exhibited rapid response and become HCV RNA negative (Santantonio et al., 2005). These results proposed that established chronic infection leads to acquire the non-response to IFN-γ. Several factors have been focused in various clinical examination of non-responders include viral strain, quasispecies and sequence divergence, complications in cell signaling and actions, the effects of co-morbidities (obesity, diabetes, renal disease and immunodeficiency), status of the disease (severity and activity of hepatitis), pharmacokinetic profiles (drug and dosage), and possibly variable environmental factors (alcohol, smoking and adjunctive medications). A profound impact of host innate and adaptive immunity has been observed HCV infection (Kenny-Walsh, 1999). Earlier, it was reported that impaired host immunity in particular cellular immune response may lead to chronic HCV infection (Dolganiuc et al., 2006; Marukian e al., 2008 and Shiina and Rehermann 2008). It has also been observed that in different hosts, genetic factor(s) may be responsible for the distinct consequences of HCV infection (Thio et al., 2000 and Missiha et al., 2008).

Various genome-wide association studies (GWAS) have independently revealed that variations (in particular rs12979860) in IL28B (interleukin- 28B), are strongly associated with the natural defense of host against HCV infections in western populations (Thomas et al., 2009). Moreover, their association with HCV patients' response to pegylated-IFNa and RBV treatment was also observed in different populations (Suppiah et al., 2009 and Tanaka et al., 2009). IL28B gene in the host encodes cytokine IL-28B (IFNl3) (Ank et al., 2006) and belongs to the type III IFN family which is also known as IFN-lamdas (IFNλs). IL-29 (IFNλ1) and IL-28A (IFN λ2) are also members of this family (Sheppard et al., 2003 and Sommereyns et al., 2008). The genes of IFNλs are clustered on human chromosome 19. IL-28B is the latest member of IFN family and plays a critical role in clearing viral infections (Kotenko et al., 2003 and Doyle et al., 2006). It is mainly produced by macrophages and dendritic cells (DCs) in response to the stimuli of viral proteins or toll-like receptor agonists (Ank et al., 2008). IL-28B plays an important role in antiviral responses including HCV infection (Thomas et al., 2009). IFNλs signal via common type III interferon receptors consisting of IL- 10R2 and IL-28R subunit and trigger JAK-STAT (Janus kinase signal transducers and activators of transcription) pathway

to induce the expression of IFN-stimulate genes (ISGs) (Stark et al., 1998). These ISG products can subsequently suppress a wide range of viral replications and protein synthesis including hepatitis B and C virus (Samuel, 2001). IFNλ receptors are expressed on a variety of cells, predominantly the liver and epithelial cells (Sheppard et al., 2003 and Spann et al., 2005), suggesting that they may have a profound impact on liver function. IL28B gene was reported to be strongly associated with spontaneous (Thomas et al., 2009 and Rauch et al., 2010) and treatment-induced clearance of HCV (Ge et al., 2009; Suppiah et al., 2009 and Tanaka et al., 2009) although the functional link between IL28B polymorphisms and HCV clearance remains elusive. It has also been observed that type III IFNs exhibit *in vivo* (Muir et al., 2010) and *in vitro* (Robek et al., 2005 and Marcello et al., 2006) antiviral activity against HCV. A classical antiviral state generated by type III IFNs through mechanisms similar to, but independent of, type I IFNs may contribute to host defenses (Kotenko et al., 2003), however, most of their antiviral properties are dependent on the appropriate stimulation of the host immune system (Ank et al., 2006). It has been reported that IL28B is capable of establishing a robust T cell adaptive immune response (Morrow et al., 2009, and 2010), which may be relevant because a proper stimulation of the CD8+ response has been shown to envisage rapid and sustained virological response to therapy (Pilli et al., 2007). As a consequence, IL28B polymorphisms associated with viral persistence and poor responsiveness to therapy of HCV infection may be the hallmark of an inappropriate/ impaired activation of the adaptive immune response.

1.6 PHYLOGENETICS OF HCV

Adaptive selection and genetic drift are the two main sources of HCV evolution, a highly dynamic process (Budowle et al., 2005). During the course of evolution HCV diversification had been observed to occur and at different rates. Evolution of HCV in patients who acquired disease from a common source emphasize that host immune responses are pronounced in determining quasispecies transmission (Henderson, 2003). Contrary to the high rate of HCV mutation the evolution of HCV appeared to be conservative. Fundamental changes in HCV genotype relationship to its host are obvious and alterations in treatment response among genotypes are very important clinically e.g., their ability to flourish during long periods of evolution

thereby successfully filling specific ecological niche in human populations (Araujo et al., 2008).

1.7 OBJECTIVES

Being the highly persistent human viruses, HCV is becoming one of the major reasons of many deaths. Currently the world is facing the resistance of HCV to antiviral therapy and treatment is getting more and more complex for the physicians and researchers. Here in the present study the virus is being focused in response to the therapy and it was tried to find out that what are ways followed by this virus to escape the treatment response and which host biochemical marker helps it in escaping from treatment. Besides this a little preliminary effort has also been carried out to design the siRNA against the resistant HCV strains using *in silico* tools. The key points of the objectives are;

- To study the rationale of HCV response or non-response to antiviral therapy by evaluating
  - Serum biochemical markers
  - Viral genotypes
  - Viral genetic heterogeneity

MATERIALS AND METHODS

Being the highly persistent and causative agent of various liver diseases (inflammation, dysfunction, shrinkage and HCC), HCV is becoming a global burden now a days. Drug resistance of hepatitis C is becoming a challenge for medical practitioners and researchers these days. To reduce the present barriers to HCV treatment, it is required to recognize the contribution of cellular and antibody immune responses to viral clearance and further viral and host factors that prevent viral clearance. Research in this field is obligatory to design the preventive and therapeutic measures to plan different treatment combinations that might help the immune response together with inhibition of viral replication. Therefore in the present study we have described the association of viral heterogeneity, evolutionary dynamics and some other factors like biochemical markers with antiviral therapy.

2.1 STUDY DESIGN AND PATIENT'S SELECTION

The present study is a type of case control study included both negative and positive control groups along with the experimental groups. Initially 500 patients were recruited who were anti-HCV antibodies positive and also meet the criteria of patient selection in the present study.

2.1.1 Exclusion and Inclusion Criteria

To find the relation of the viral genetic heterogeneity, seroepidemiology and evolutionary dynamics with antiviral therapy the patients selected for the study were HCV ELISA positive and attending the out patient's department (OPD) of Pakistan institute of medical sciences Islamabad (PIMS), combined military hospital (CMH) Rawalpindi, military hospital (MH) Rawalpindi and Fauji Foundation hospital (FFH) during the period of 2010-2011. Patients belong to different geographical locations of Pakistan. All patients were receiving injections of PegIFN-α once each week plus oral RBV daily for 24 weeks as prescribed by their physician. Doses of PegIFN-α were adjusted according to the platelet and white blood cells counts of the patient. Moreover, the doses of ribavirin varied according to the weight and the hemoglobin level of individual.

The age group selected for the study was between 20-65 years. Out of 500 patients, 451 started the therapy. Before the start of therapy viral load was determined in all patients. Patients did not lie in the study age group were excluded from the

study. Also the HCV positive patients with history of alcohol, or positive for HBV or HIV antibody were not considered for the study.

2.1.2 Grouping of Patients

All the 451 patients were followed till the end of therapy. Two groups were defined on the basis of their treatment response: group 1 including the patients who exhibited SVR and group 2 constitutes the patients who were non-responders. Among the 451 patients, 376 exhibited the SVR while 75 patients remain non-responders. For serochemical analysis positive and negative control groups were also included in the study (Table 2.1).

Table 2.1: Grouping details of patients in the study.

Groups	Disease Status	Treatment Response
Group 1 (n=376)	ELISA Positive	SVR Become HCV negative after 12 weeks of treatment.
Group 2 (n=75)	ELISA Positive	Non-responders Still HCV positive even after 24 weeks of treatment.
Positive Control (n=75)	ELISA Positive	Freshly diagnosed and not receiving any therapy.
Negative Control (n=75)	ELISA Negative	Healthy subjects

2.1.3 Collection of Demographic Data

Demographic data of all the 500 patients was collected that include age, gender, history of disease, educational levels, occupations, daily work activity (sedentary or non-sedentary), and health behaviors (smoking and alcohol consumption) etc. In the proforma demographics (age, sex, educational levels, occupations), daily work activity (sedentary or non-sedentary), and health behaviors (smoking and alcohol consumption) etc were taken into consideration (Annexure I).

2.1.4 Blood Sample Collection

Patients in the treatment groups were on follow up from the diagnosis of the infection till 24 weeks of the treatment. Blood samples (2-3 ml) were also collected from positive and negative control groups. Scheme followed for sample collection is given in table 2.2.

Table 2.2: Blood sampling and antiviral treatment.

Studied Groups	Timings of Sample Collection
Group 1 (n=376)	Before treatment, after 4 weeks, after 12 weeks, after 24 weeks and after 6 month of the end of therapy.
Group 2 (n=75)	Before treatment, after 4 weeks, after 12 weeks, after 24 weeks and after 6 month of the end of therapy.
Positive Control Group (n=75)	Before the start of any therapy.
Negative Control Group (n=75)	Healthy subject's blood was taken only once.

2.1.5 Storage of the Blood Samples

The aspirated blood was divided into two tubes as to perform both molecular and biochemical analysis. For molecular analysis the blood was shifted to K_2.EDTA tubes and for biochemical analysis it was shifted to silica gel tube. Blood in both types of tubes were centrifuged for the separation of the sera. The serum was stored at -80 °C for further analysis.

2.2 RNA EXTRACTION FROM SERUM SAMPLES

Viral RNA was extracted using commercially available kit (NucleoSpin RNA Virus). The preparation of reagents was managed according to the manufacturer's instructions. Before the start of the extraction protocol, following regents were prepared.

RAV1: Before use, 1 ml of lysis buffer RAV1 was added to the carrier RNA tube and was mixed thoroughly and transferred back to RAV1 bottle.

Wash buffer RAV3: In the bottle of RAV3 buffer, 1 ml of ethanol (96-100 %) was added and mixed properly before use. The protocol was performed as:

For the lyses of virus 600 µl buffer RAV1 containing Carrier RNA was added to 150 µl of the sample. Mixture was pippetted up and down and vortex well followed by incubation at 70 °C for 5 minutes.

- To adjust the binding conditions 600 µl ethanol (96-100 %) was added to the clear lyses solution and mixed by vortexing (10-15 seconds).

- For viral RNA binding Nucleospin RNA Virus Columns were placed in collection tubes (2 ml) and 700 µl lysed sample was loaded followed by centrifugation for 1 minute at 8,000 x g. After discarding the flowthrough the column was positioned in another collection tube (2 ml) while the remaining residual lysed solution was loaded and centrifuged for 1 minute at 8,000 x g.
- After viral RNA binding three washing steps were performed.
  - In first wash 500 µl RAW buffer was added to the column, centrifuged for 1 minute at 8,000 x g and flow-through was discarded.
  - For second wash 600 µl RAV3 buffer was added to the column. Centrifuged for 1 minute at 8,000 x g and flow-through was discarded again.
  - For last wash column was placed in a new collection tube and 200 µl RAV3 buffer was added and centrifuged for 2-5 minutes at 11,000 x g to remove ethanolic RAV3 buffer completely.
- To elute viral RNA, column was placed into a new sterile 1.5 ml microcentrifuge tube and 50 µl RNase-free H_2O (preheated at 70 °C) was added and incubated for 1-2 minutes followed by centrifugation for 1 minute at 11,000 x g. The resulting solution contained the viral RNA that was further processed for cDNA synthesis.

2.2.1 Measurement of Viral Load in ELISA Positive HCV Patients

Quantification of HCV RNA was done using sequence detection system GeneAmp 5700 (Applied Biosystems) with HCV RNA quantification kit (Superscript™ 1-Step RT-PCR Kit with Platinum® *Taq* Invitrogen). The whole process was performed in a single step including reverse transcription and quantitative PCR (qPCR) amplifications in triplicate using 96-well plates. The components of reaction mixture are given below:

Components	**Concentrations and Volumes**
2X Reaction Mix	25 µl
Template RNA	10 pg – 1 µg
Sense Primer (10 µM)	1 µl 0.2 µM
Anti-sense Primer (10 µM)	1 µl 0.2 µM
RT/ PlatinumR *Taq* Mix	200 U
Autoclaved Distilled Water	up to 50 µl
Final Concentration	50 µl

Initially the reaction mix was heated at 50 °C for 60 minutes to facilitate cDNA synthesis. *Taq* polymerase was activated at 95 °C followed by 10 min incubation and denatured the DNA template and reverse transcriptase. Forty-five cycles were carried out (included denaturation for 30 s at 95 °C, annealing for 15 s at 65 °C and finally the extension for 1 min at 72 °C) and then permitting the parallel amplification and quantification of the HCV fragments. HCV RNA was quantified in IU/ml, by comparing the results for the unknown serum samples to those on a standard curve. Besides the negative controls, an aliquot of 100 µl for each of the standards was separately detected after extraction and amplification and were run in parallel with the samples being tested. At the end of each reaction a standard curve was also generated by plotting the threshold cycle (C_T) (the cycle at which a fluorescence signal above baseline can be detected) against $\log_{10}(N)$, where N is the initial concentration of the standard in IU/ml. HCV RNA concentrations for all unknown samples were subsequently calculated by interpolation of the standard curve.

2.3 BIOCHEMICAL PROFILING

Biochemical profiling was performed at the time of patient's visit to physician i.e before the start of treatment, at 4th week, 12th week, 24th week and after 6 months of the end of therapy. Blood was aspirated at their follow up visit to the physician and was proceeded for the liver function test, lipid profile and hematological indices. All tests were performed using commercially available reagent kits.

2.3.1 Liver Function Test

Liver enzymes; Alanine Amino Transferase (ALT), Aspartate Amino Transferase (AST) and Bilirubin were estimated through visible light spectrophotometer (UV 4000 Spectrophotometer, O.R.I. Germany). Commercially available reagent kits (AMP Diagnostics) were used. The reagents were prepared for each test according to the manufacturer's instructions.

2.3.1.1 Alanine Amino Transferase (ALT)

The enzyme alanine aminotransferase (ALT) catalyzes the transaminase reaction between L-alanine and 2- oxoglutarate. The pyruvate thus formed, is reduced to lactate in the presence of Lactate dehydrogenase (LDH). As the reactions proceed, NADH is oxidized to NAD. The disappearance of NADH per unit time is followed by measuring the decrease in absorbance at 340 nm.

- **Protocol**

Initially 4 volumes of reagent R1 were mixed with 1 volume of reagent R2. All the samples, reagents and control were incubated to reaction temperature. The photometer was adjusted to zero with distilled water. Working reagent (1 ml) and serum sample (50 µl) were first mixed together and incubated at 37 °C for 1 minute and initial absorbance was recorded at 340 nm. Absorbance was recorded three times exactly after 1 minute of the previous reading. Final results were calculated using formula given below and average absorbance of three readings was used to calculate the final results. Absorbance reading exceeded 0.160 was reassayed and also diluted 1:10 with saline. The final results were multiplied by 10.

$$\text{ALT activity [U/L]} = \Delta A/\text{min} \times 3333$$

2.3.1.2 Aspartate Amino Transferase (AST)

The presence of aspartate aminotrasferase (AST) catalyzes the trasamination reaction between alpha ketoglutaric acid and L-aspertate acid, causing the formation of L-ketoglutamic acid and oxaloacetic acid, in the presence of malate dehydrogenase, (MDH), reacts with NADH forming malic acid and NAD^+ in equal quantities. The resulting rate of decrease in absorbance at 340 nm is directly proportional to AST activity. LDH is added to prevent interference from endogenous pyruvate which is normally present in serum.

- **Protocol**

Two reagents R1 and R2 were mixed together in a ratio of 4:1 and used as working reagent. This freshly prepared working reagent (1 ml) and serum sample (50 µl) were first mixed together and incubated at 37 °C for 1 minute and initial absorbance was recorded at 340 nm. An average of three readings was used to calculate the final results using following formula. Samples were diluted in a ratio of 1:10 with saline and reassayed when the absorbance reading exceeded the 0.160.

$$\text{AST activity [U/L]} = \Delta A/\text{min} \times 3333$$

2.3.1.3 Total Bilirubin (TBL)

Total bilirubin reacts with diazotized sulphanilic acid and caffine to give a colored azocompound.

- **Protocol**

Working reagent was prepared by adding 10 µl of R2 to 1 ml of R1 and mixed properly before use. Sample (50 µl) and working reagent (1 ml) were mixed together

and incubated at 37 °C for 1 minute. Absorbance was recorded at 540 nm. Absorbance was taken in thrice and an average absorbance was put in the formula given below to get the final result.

$$\frac{\text{Absorbance of the sample - Absorbance of the bl}}{\text{Absorbance of the standard}} \times C_{\text{standard}}$$

2.3.1.4 Determination of Serum Proteins

Serum proteins have very important roles to play in the repair and maintenance of body tissue. In the present study total serum proteins and serum albumin concentration was determined.

- **Protocol**

The working reagents provided were ready to use. Serum sample (10 µl) was mixed with working reagent (1 ml) and incubated at 37 °C for 10 minutes. Optical density of serum sample mix with reagent, and also of standards was measured at 550 nm. Following formula was used to calculate the final readin:

$$\frac{\text{Absorbance of the sample}}{\text{Absorbance of the standard}} \times n$$

(n = standard concentration)

2.3.1.5 Measurement of Serum Albumin

The method is based on the specific binding of bromocresol green (BCG), and anionic dye, and the protein at acidic pH with the resulting shift in the absorption wavelength of the complex. The intensity of the colour formed is proportional to the concentration of albumin in the sample.

Protocol

Serum sample (10 µl) was mixed with working reagent (1.5 ml) and incubated at 37 °C for 1 minute. Absorbance of the sample and standard was taken against the blank reagent at 630 nm. The final result was obtained using following formula:

$$\text{Albumin (g/dl)} = \frac{\text{Absorbance of the sample}}{\text{Absorbance of the standard}} \times 4$$

2.3.2 Lipid Profile

As liver perform the vital role in lipid metabolism it is of great significance to determine the lipid profile of the patient with liver disease. In the current study serum cholesterol, triglycerides, high density lipids (HDL) and low density lipids (LDL)

were measured using the commercially available kits (AMP, diagnostics). All the reagents provided in the kits were ready to use. Principal and protocols of each technique is given below;

2.3.2.1 Total Cholesterol

All cholesterol esters present in the plasma are hydrolysed quantitatively into free cholesterol and fatty acids by cholesterol esterase. In the presence of oxygen, free cholesterol is then oxidized by cholesterol oxidase to cholest – 4 – one-3-one and hydrogen peroxide. The hydrogen peroxide reacts with phenol and 4 aminoantipyrine in the presence of peroxidase to form a coloured dye. The intensity of the colour formed is proportional to the cholesterol concentration and can be measured photometrically between 480 and 520 nm.

- **Protocol**

Working reagents and standard were incubated at 37 °C. The absorbance of the serum sample (10 µl) and standard (10 µl) against the reagent blank (1000 µl) was measured within 60 minutes. The results were calculated as under:

$$\frac{A_{sample}}{A_{standard}} \times C_{standard} = \text{mg/dl cholesterol}$$

2.3.2.2 Estimation of Serum Triglycerides

The triglycerides in the sample are hydrolyzed enzymatically by lipase to glycerol and fatty acids. The glycerol formed is converted to glycerol phosphate by glycerol kinase. Glycerol phosphate is oxidized to dihydroxyacetone phosphate by glycerol phosphate oxidase. The liberated hydrogen peroxide is detected by a chromogenic acceptor, chlorophenol-4 aminoantipyrine, in the presence of peroxidase. The red quinone formed is proportional to the amount of triglycerides present in the sample.

- **Procedure**

Reagents with the given constituent concentrations present in the kit were ready to use. The reagents were incubated for 5 minutes at 37 °C. Then the absorbance of unknown sample (10 µl) and standard (10 µl) was measured at 500 nm against blank reagent (1 ml). The results were calculated as under:

$$\frac{A_{sample}}{A_{standard}} \times 200 = \text{Triglycerides (mg/dl)}$$

2.3.2.3 Determination of High Density Lipoproteins Cholesterol (HDL-C)

Anti-human β-lipoprotein antibody in R1 binds to lipoproteins (LDL, VLDL) other than HDL. The antigen- antibody complexes formed block enzyme reactions when R2 is added. Cholesterol estrases (CHE) and cholesterol oxidase (CO) in R2 react only with HDL-C. Hydrogen peroxide produced by the enzyme reactions with HDL-C yields a blue color complex upon oxidative condensation of F-DAOS [(N-ethyl-N-(2-hydrixy -3-sulfopropyl)-3, 5-dimethoxy-4-fluoroani-line, sodium salt) and 4-aminoantipyrine (4-AA) in the presence of peroxidase (POD).

- **Protocol**

Serum sample (240 µl) was mixed gently with working reagent (240 µl) and incubated for 4 minutes at 37 °C. Absorbance was recorded at the wavelength of 600 nm for sample and calibrator against the blank. Following formula was used to get the final reading:

$$\frac{\text{Absorbance of the sample}}{\text{Absorbance of the calibrator}} \times C_{\text{calibrator}}$$

2.3.2.4 Deterination of Low Density Lipoprotein Cholesterol (LDL-C)

LDL was measured indirectly using the Friedewald equation, a mathematical formula developed by William Friedewald to calculate the concentration of LDL, or "bad" cholesterol, in the bloodstream. This equation calculates the concentration of LDL based upon the presence of total cholesterol, HDL and triglyceride levels:

LDL = total cholesterol – HDL – (triglycerides/5)

2.3.3 Hematological Indices

Hematological abnormalities including neutropenia, anemia, and thrombocytopenia have been reported in HCV patients treated with PegIFN and ribavirin. In the preset study the hemoglobin, platelet count and prothrombin time is under observation.

2.3.3.1 Blood Counts through Hematology Analyzer

Blood collected in the vacutainers was immediately processed for the determination of hemoglobin and platelet count on hematology analyzer (Sysmax KX-21 Japan).

2.3.3.2 Estimation of Prothrombin Time

Prothrombin time was estimated using reagent kit "Thromboplastin with calcium" (Biomerieux Corp. France). The reagent contained lyophilized extract of

rabbit brain with buffer, stabilizer and calcium chloride. The extract contained thromboplastin that in the presence of calcium ions accelerates the blood clotting by the activation of extrinsic pathway of coagulation. The reagent was reconstituted with the addition of 2 ml deionized water. It was left for approximately 30 minutes at room temperature with occasional swirling. The citrated plasma was kept at 37 °C for at least 15 minutes in the water bath. Plasma (50 µl) was added in a torpedo tube and incubated at 37 °C for 60-180 seconds, 100 µl reagent was then added to the plasma and time was noted in seconds until coagulation of the plasma.

2.3.4 Statistical Analysis

After performing all the biochemical assays, statistical analysis was performed. Descriptive statistical analysis was carried out with software Stata version 12. Box and whisker plots were prepared for all variables. The difference in proportions were estimated by Bartlett's tests. A p-value of < 0.05 was considered to indicate statistical significance. Univariate analysis was applied on variables including viral load, ALT, AST, TBL, TCOL, HDL, LDL, Hb level, PLT and PTT. The treatment groups were compared for each variable using LSD test. A p-value of $<$ 0.01 was considered to indicate statistical significance for LSD. All univariate analysis was performed using Co-Stat 6.311. Pearson co-efficient of correlation between HCV viral load and other biochemical descriptors were calculated using STATISTICA version 8.0. Scatter biplots for important correlations were prepared for better visulation of relationship between HCV viral load and other variables.

2.4 GENETIC AND MOLECULAR ANALYSIS OF HCV

Genome of HCV is responsible for its infectious nature. Each genomic region has its own role in the pathogenesis of HCV. In the current study three genomic regions including 5'UTR, core and NS5B have been analyzed for their role in response to antiviral therapy in HCV infected patients. Randomly picked 75 patients from group 1 were compared with 75 non-responders that were represented by the group 2.

2.4.1 Primer Designing

Primers were designed in nested manner with external and internal sets for each genomic region of the present study in HCV genome. Available sequences of HCV in the GenBank were obtained to design specific primers using online available

tool Primer 3 for the amplification of partial regions of core, 5′ UTR and NS5B. Sequences of the designed primers are given below (Table 2.3).

Table 2.3: Nucleotide sequences of the designed primers.

PCR	Genomic Region	Sequence of Primers
Nested	5′UTR	SP1: 5′CTGTGAGGAACTACTGTCTT 3′ ASPI : 5′ATACTCGAGGTGCACGGTCTACGAGACCT 3′ SP2: 5′TTCACGCAGAAAGCGTCTAG 3′ ASP2: 5′CACTCTCGAGCACCCTATCAGGCAGT 3′
Nested	Core	SP1: 5′ ACTGCCTGATAGGGTGCTTGC 3′ ASP1: 5′ ATGTACCCCATGAGGTCGGC 3′ SP2: 5′ AGGTCTCGTAGACCGTGCAC 3′ ASP2: 5′ CACGTAAGGGTATCGATGAC 3′
Semi Nested	NS5B	SP1: 5′ GCCTTCACGGAGGCTATGAC 3′ ASP1: 5′ GGCACCCAAGCTTTCTGAG 3′ ASP2: 5′ ACACGCTGTGATAAATGTC 3′

2.4.2 Complimentary DNA (cDNA) Synthesis

RT-PCR is commonly used in studying the genomes of viruses whose genomes are composed of RNA (Mackay et al., 2002). The extracted RNA (10 µl) was reverse transcribed into complementary DNA (cDNA) with reverse transcriptase enzymes like Maloney Maurine Leukemia Virus (M-MLV reverse transcriptase enzyme) (Fermentas). The composition of reaction mixture and cycling conditions were as follow:

2.4.2.1 Reaction and PCR Conditions for cDNA Synthesis

Components	Concentrations and Volumes
DEPC Treated Water	6 µl
5X RT Buffer	4 µl
10 mM dNTPs	2 µl
RNase Inhibitor	40 U
Oligo ASP1	1 µl
MMuLV Reverse Transcriptase	200 U
Template RNA	5 µl
PCR Conditions	
37 °C	5 minutes

42 °C	60 minutes
95 °C	5 minutes

2.4.3 Nested Polymerase Chain Reaction (PCR)

Nested PCR is advisable for viruses like HCV and it was carried by using respective primers of all three genomic regions under consideration in this study. This is a sensitive and accurate method for the amplification of HCV from blood. One negative and one positive control (already known positive HCV sample) was run to ensure the reliability of the results. Same protocol was used to amplify all the three genomic regions with gene specific external and internal set of primer. Annealing temperature varied according to the primers used.

2.4.3.1 Reaction and PCR Conditions for Nested PCR

External PCR

Components	**Volumes**
Sterowater	14.125 µl
10X PCR Buffer	2.5 µl
10 mM dNTPs	0.5 µl
10 pM/µl SP1	0.625 µl
10 pM/µl ASP1	0.625 µl
Taq DNA Polymerase	0.125 µl
10 mM $MgCl_2$	1.5 µl

PCR Conditions:

94 °C	5 minute	
94 °C	1 minute	40 Cycles
* °C	1 minute	
72 °C	1 minute	
72 °C	5 minutes	
4 °C	∞	

* °C: 58°C for 5'UTR
60°C for Core
55°C for NS5B

Internal PCR

Components	Volumes
Sterowater	14.125 µl
10X PCR Buffer	2.5 µl
10 mM dNTPs	0.5 µl
10 pM/µl SP2	0.625 µl
10 pM/µl ASP2	0.625 µl
Taq DNA Polymerase	0.125 µl
10 mM $MgCl_2$	1.5 µl

PCR Conditions:

94 °C	5 minutes	
94 °C	1 minute	40 Cycles
* °C	1 minute	
72 °C	1 minute	
72 °C	5 minutes	
4 °C	∞	

*°C: 60°C for 5'UTR
56°C for Core
58°C for NS5B

2.4.4 Gel Electrophoresis of the Amplified Products

Gel electrophoresis was performed by mixing PCR product with 6X loading dye (Bromophenol blue). The PCR products and 50 bp DNA ladder (Fermentas) were electrophorized using 1.5 % agarose gel prestained with ethidium bromide in 0.5 X TAE (Tris-Acetate-EDTA) buffer. The gel was finally visualized in a gel documentation system (Wealtech-Dolphin-Doc^{Plus}).

2.4.5 PCR Product Purification

Amplified product was purified using Jet quick (PCR product purification spin Kit by Genomed) by following the instructions given by the manufacturer.

- **Sample Preparation for Purification**

  200 µl of solution H1 (Guanidine hydrochloride and isopropanol) was added to 40 µl of PCR product and was mixed thoroughly.

- **Column Loading**

The mixture from step1 was loaded into the Jetquick spin column placed in a 2 ml receiver tube followed by spinning for 1 minute at 13,000 rpm (using Spectrafuge 24D Labnet) and flow through was discarded.

- **Column Washing**

  Reconstituted H2 solution (ethanol, NaCl, EDTA and Tris HCl) was added to spin column inserted in fresh receiver tube. The tube was spinned for 1 minute at 13,000 rpm. Before using H_2 solution sequencing grade ethanol (96-100 %) was added to its concentrated buffer. After discarding the flow through, the column was repositioned into the same receiver tube and centrifuged for 1 minute at 13,000 rpm.

- **DNA Elution**

  JetQuick spin column was placed into a fresh 1.5 ml microfuge tube and 30 µl of Nanopure water was added (or TE buffer or 10 mM tris/HCl, pH 8.0) straight onto the core of silica medium of the JetQuick spin column. The tube was centrifuged at 13,000 rpm for 2 minute. Elution buffer (preheated to 65-7 °C) was added straight in the middle of the silica matrix of the spin column and was allowed to stand 1 minute prior to spinning. Elution was dispensed directly onto the silica membrane. After centrifugation DNA was stored for further use.

2.4.6 Sequencing

Purified samples of HCV were sent to Macrogen, South Korea for DNA sequencing.

2.4.7 Sequence Homology

Basic Local Alignment Search Tool (BLAST) was used to find regional homology among all the studied sequences and already reported sequences (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The obtained sequences of the present study were pasted in FASTA format and searched for maximum similarity with already reported HCV sequences.

2.4.8 Sequence Submission to GenBank

Sequence data is being submitted to GenBank and accession numbers awaited.

2.4.9 Relationship of Genotypes with Demographics of Patients

Demographic characteristics of patients i.e., gender, age and mode of transmission were evaluated in relation to HCV genotypes in patients of both the groups 1 and 2.

2.5 EVOLUTIONARY DYNAMICS AND HCV RESPONSE TO ANTIVIRAL THERAPY

To track the evolutionary pattern, genetic heterogeneity and mutation pattern of HCV, different bioinformatics tools were used to proceed *in silico* analysis.

2.5.1 Determination of Phylogeny

MEGA5, online available software was used to check the genetic diversity level of HCV sequences in response to antiviral therapy. This tool infers phylogenetic trees, estimating rates of molecular evolution, inferring ancestral sequences, and testing evolutionary hypotheses (Nei and Kumar 2000 and Tamura et al., 2011). Multiple aligned sequences were uploaded in MEGA5 and maximum parsimony method was used to construct phylogenetic tree of all the three concerned genomic regions of the present study in comparison to the reference sequences obtained from database from the whole world.

2.5.2 Sequence Diversity of 5'UTR, Core and NS5B

An online available tool BioEdit version 7.3 was used to determine the sequence diversity at nucleotide level. Rate of insertions, deletions, transversions and transitions in the nucleotide sequences were determined using this software. Besides this mutation spots and number of synonymuous and non-synonymous substitutions were also calculated.

2.5.3 Translation of Nucleotide Sequences into Amino Acid Sequence

Nucleotide sequences were required to be converted into amino acid sequences for 3D structural analysis. Online available tool TRANSLATE was used for translation (http://hcv.lanl.gov/content/sequence/TRANSLATE/translate.html).

2.5.4 3D Structural and PROCHECK Analysis

For 3D structural analysis, models were built using i-TASSER (Roy et al., 2010) and were evaluated on PROCHECK. It checks the stereo-chemical quality of a protein structure, producing a number of PostScript plots analyzing its overall and residue-by-residue geometry (http://www.ebi.ac.uk/thornton-srv/software/PROCHECK/). 3D models pdb files were uploaded and Ramachandran

Plot was used to check the existence of five models predicted by i-TASSER for each sequence.

2.5.5 Analysis of Amino Acid Heterogeneity

Amino acid heterogeneity analysis was done by aligning amino acids of both core and NS5B using Bioedit clustal W programme. These aligned sequences were then analyzed using MEGA version 5 (Tamura et al., 2011). Number of conserved sites, number of variable sites and amino acid composition was calculated for each sample of both groups and then comparisons were made between the two groups. Amino acid substitution was determined using online available software DNAman.

2.5.6 Determination of N-Linked Phosphorylation Site

Online available web server NetPhos 2.0 was used that produces neural network predictions for serine, threonine and tyrosine phosphorylation sites in proteins. Phosphorylation sites were predicted for both the proteins core and NS5B for all the 75 patients each from both groups (1 and 2).

2.5.7 Determination of N-Linked Glycosylation Sites

To predict the N-glycosylation sites, the amino sequences of core and NS5B were uploaded on the online availabe tool SignlN-PP. The predicted sites crossing the threshold were counted and compared among the two study groups.

2.6 Computational Approach for siRNA Prediction for 5' UTR

Antiviral siRNA prediction was made for HCV sequences. siRNA sequences were selected based on their degree of conservation, defined as the proportion of viral sequences that are targeted by the corresponding siRNA, with complete matches. The tool used for prediction of siRNA was siDirect (http://genomics.jp/sidirect/) a highly effective, target specific siRNA online designed tool. HCV sequenced samples were pasted in Fasta format and the program was run using algorithm that is on the back hand of siDirect, which is Ui-Tei algorithm (Ui-Tei et al., 2004). The output page displays the siRNA sequence and siRNA position.

RESULTS

3.1 SEROEPIDEMIOLOGY OF HCV POSITIVE PATIENTS IN RESPONSE TO THE TREATMENT

The data was collected on HCV from Pakistan institute of medical sciences Islamabad (PIMS), combined military hospital (CMH) Rawalpindi, military hospital (MH) Rawalpindi and Fauji Foundation hospital (FFH) Rawalpindi from the patients visiting the liver OPDs with different ethnic origins from all over the Pakistan. Initially 500 patients gave consent to participate in the study. Among them 207 were females while 293 were males. Overall the ratio of the HCV positive males was high as compared to females (Figure 3.1.1) in the present study.

3.1.1 Natural History of the HCV for the Studied Cohort

The selected cohort of patients constituted of 80 % chronic cases of HCV and only 20 % acute infection. There were no apparent gender differences in the rate of chronicity in hepatitis C infection. Majority of the considered cases whether males or females were equally suffering from the chronicity of the disease (Figure 3.1.2).

3.1.2 Grouping of Patients

Blood of all the 500 patients was analyzed for viral load determination and estimation of all biochemical markers. After taking the informed consent, as the patients were proceeded with the therapy; 4 patients were died, 8 patients left the treatment due to adverse side effects, and 18 patients withdraw the study due to some of their personal reason (Figure 3.1.3). The remaining 470 patients were under consideration but later on 19 more patients were eliminated as could not followed up due to their shifting from the city or so on. The 451 remaining patients were grouped according to their response to the treatment. Viral load and all the serum biochemical parameters were estimated in blood of all the 451 patients before the start of therapy and at different intervals throughout their treatment periods. At the end of therapy overall 376 patients became HCV RNA negative and were remained negative till the 6 month after the end of therapy and their response was considered as SVR by the physician while 75 patients remain non-responders to the therapy. These patients were grouped as group 1 (with SVR) and group 2 (non-responders).

According to this grouping pattern, at the end of the study the seroepidemiological data of all the 451 patient's on follow up was compiled as group 1 (376 patients) and group 2 (75 patients) (Figure 3.1.3).

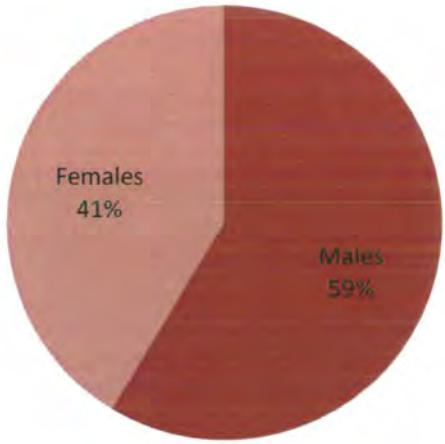

Figure 3.1.1: Gender distribution of HCV positive patients in the present study.

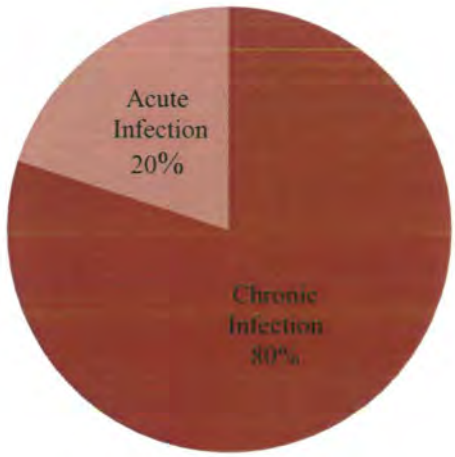

Figure 3.1.2: Distribution of patients on the basis of chronicity of disease.

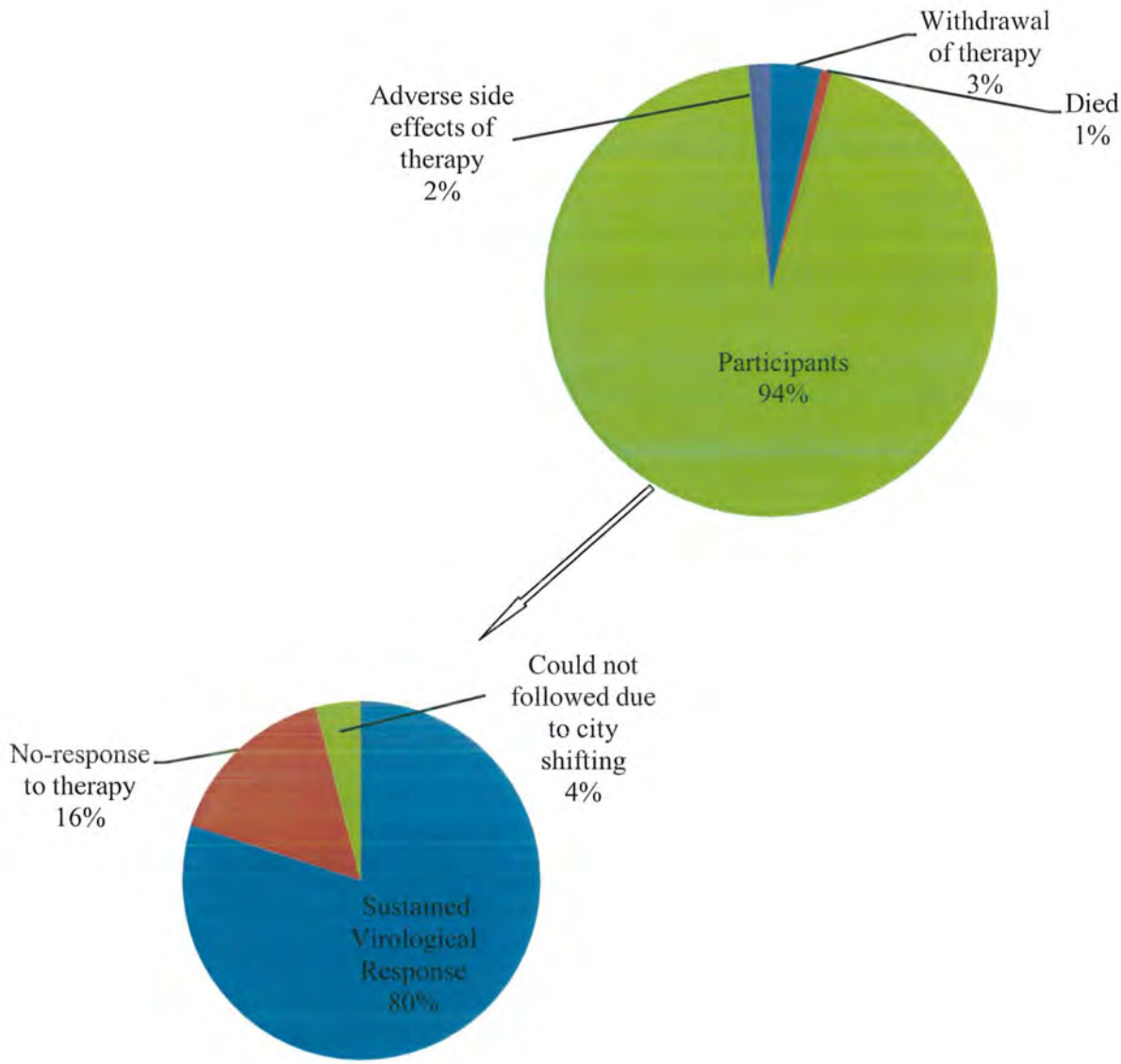

Figure 3.1.3: Patient's grouping for the study.

3.1.3 Seroepidemiology of HCV

In the present study, the serum analysis for selected biochemical markers directly related with liver disease was performed for the final participants of the study and further the correlation of the serum biochemical markers was checked with HCV viral load. The obtained results were analyzed using different statistical analysis.

3.1.3.1 Determination of Viral Load

Quantities of HCV RNA, in IU/ml, were determined by comparing the results for the unknown serum samples to those on a standard curve. In group 1 (SVR) the baseline viral load was low as compared to the group 2 (non-responders). Univariate analysis revealed that patients in group 1 (with SVR) possessing low baseline viral load showed significant decline in viral RNA copies at the start of therapy ($P > 0.000$) while become completely HCV RNA negative and remain so afterwards even when analyzed 6 months after the end of therapy. However, patients with high baseline viral load took 24 weeks treatment to become completely HCV RNA negative (Table 3.1.1). Furthermore in group 2 (non-responders), patients did not show any significant decline in their viral load till 24 weeks of treatment ($P = 0.076$) (Table 3.1.1 and Figure 3.1.4).

3.1.3.2 Comparative Analysis of LFTs with the Antiviral Therapy

LFTs for ALT, AST, TBL, total protein and albumin were performed for all the treatment groups (1 and 2) along with positive and negative control groups. Furthermore, the comparison within the groups (1 and 2) associated with the duration of treatment was also performed. In the present study, the patients constituting the group 1 (with SVR) showed significant decrease in serum ALT, AST and TBL ($P = 0.000$, $P = 0.000$ and $P = 0.000$) with the progression of therapy as compared to the serum concentrations before the start of therapy (Table 3.1.2). At 4^{th} week of treatment a substantial drop was found that sustained till 12^{th} week and even so on. A significant drop in serum concentrations of ALT, AST and TBL were shown from 92 ± 11.28 IU/L, 74 ± 14.04 IU/L and 3 ± 0.652 mg/dl to 24 ± 4.10 IU/L, 34 ± 3.02 IU/L and 0.60 ± 0.11 mg/dl respectively till 24^{th} week of treatment that become comparable to the negative control group (Figure 3.1.5).

Table 3.1.1: Univariate analysis of HCV viral load of all studied cohorts.

Treatment	95 % Confidence Interval (CI)		Mean Square (MS)	F-Value
	lower limit	upper limit		
Group 1 (n=376)				
Before the start of treatment	672.22	873.62	2.021	0.9589
After 4 weeks	18612	45911	2.0216	0.9589
After 12 weeks	101.16	442.51	Undetectable	Undetectable
After 24 weeks	14.21	144.1	Undetectable	Undetectable
6 months after the end of therapy	1.0	1.0	Undetectable	Undetectable
Group 2 (n=75)				
Before treatment	560.73	855.03	35557	3864964
After 4 weeks	2.3515	268.05	421303.15	7.9168
After 12 weeks	188.95	195.37	3272867.54	4.67111
After 24 weeks	221.79	250.21	4.4797	0.8739
Positive control (ELISA Positive)	541.55	831.41	2.5334	0.9179323

r: Correlation Coefficient

p: Statically Significant

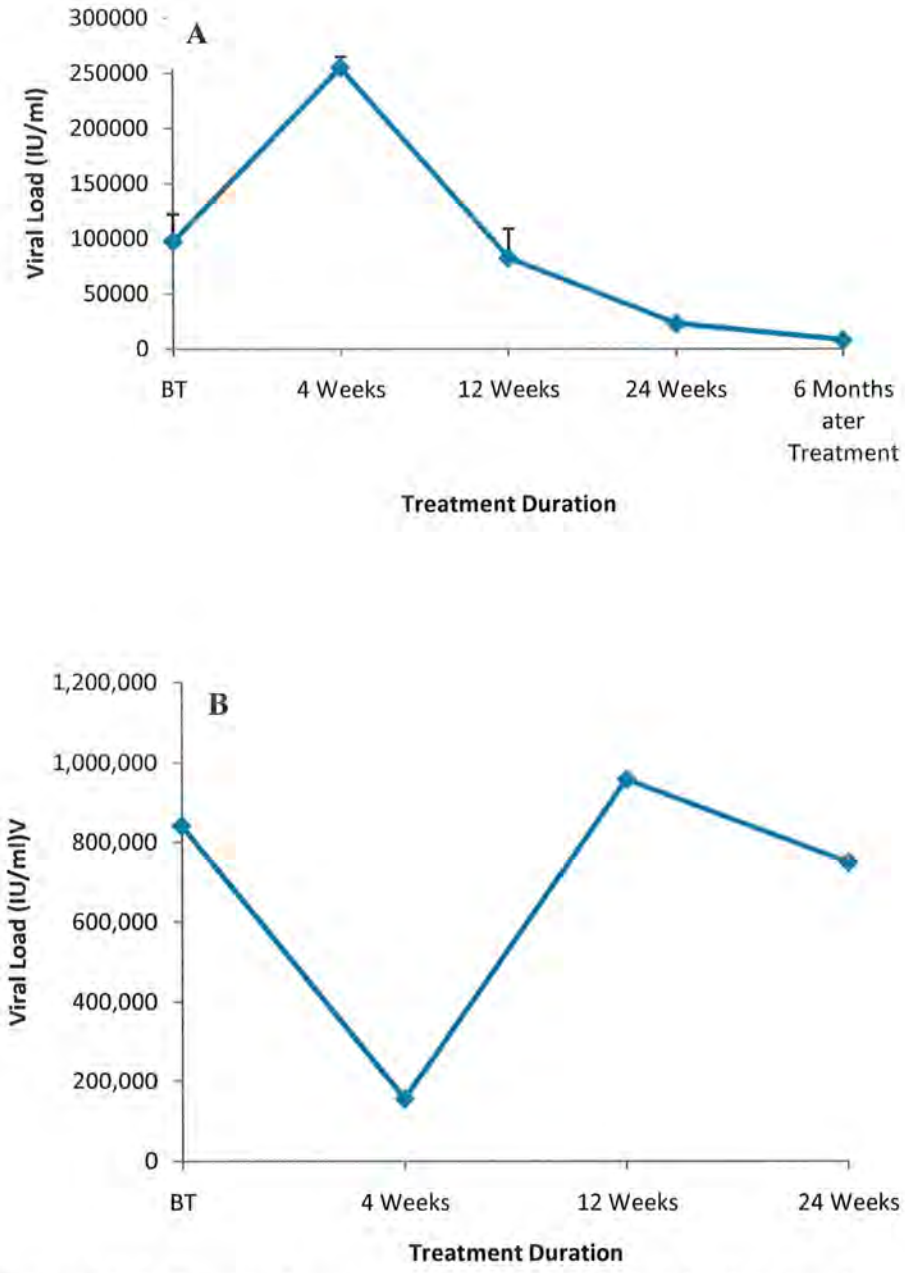

Figure 3.1.4: Graphical representation of HCV viral load during different treatment periods in both the treatment groups. (A): Group 1 (with SVR)(B): Group 2 (non-responders). (BT: Before Treatment)

Table 3.1.2: Univariate analysis of liver profile for each studied cohort.

Treatment Duration	ALT (IU/L)				AST (IU/L)				TBL (mg/dl)				Total Protein (g/dl)				Albumin (g/dl)			
	95 % Confidence Interval (CI)		Mean Square (MS)	F-Value	95 % Confidence Interval (CI)		Mean Square (MS)	F-Value	95 % Confidence Interval (CI)		Mean Square (MS)	F-Value	95 % Confidence Interval (CI)		Mean Square (MS)	F-Value	95 % Confidence Interval (CI)		Mean Square (MS)	F-Value
	lower limit	upper limit			lower limit	upper limit			lower limit	upper limit			lower limit	upper limit			lower limit	upper limit		
Group 1 (n = 376)																				
Before treatment	87.26	96.58	381.7	15.3	68.32	79.92	592.1	23.7	2.371	2.909	1.3	127.8	12.63	13.6	4.1	0.453	5.987	6.621	1.8	0.442
4 weeks	37.86	41.8	381.7	15.3	51.39	59.97	592.1	23.7	1.089	1.551	1.3	127.8	12.69	13.6	13	0.453	4.972	5.564	1.8	0.442
12 weeks	28.87	31.77	37.18	1.49	37.62	45.1	246.5	9.86	0.858	1.183	0.5	46.5	8.16	9.712	11	1.178	4.678	5.114	0.8	0.209
24 weeks	24.91	28.29	50.5	2.02	33.67	36.17	27.48	1.1	0.559	0.657	0	1.375	7.4288	8.475	4.8	0.536	4.633	5.127	1.1	0.269
6 months after the end of therapy	30.08	32.56	27.18	1.09	30.75	34.37	57.77	2.31	0.572	0.692	0.1	6.43	6.9063	7.478	1.4	0.16	4.46	4.788	0.5	0.118
Group 2 (n = 75)																				
Before treatment	71.9	81.7	214.1	8.56	89.7	94.3	248	9.92	4.89	4.95	0.6	55.62	54.226	58.41	25	2.73	6.05	6.75	0.5	0.115
4 weeks	58.5	73.3	100.5	4.02	140.6	160.5	77.18	3.09	1.31	1.51	0.7	71.88	18.6	19.65	5.9	0.657	4.99	5.33	0.4	0.099
12 weeks	72.3	83.1	52.25	2.09	6.786	7.11	85.5	3.42	6.61	6.914	0.6	62.83	9.8782	10.91	5.2	0.576	9.95	11.04	0.5	0.121
24 weeks	89.2	96.3	23.13	0.93	162.5	176.4	47.25	1.89	5.82	5..97	0.5	47.67	26.76	30.52	4.7	0.525	8.73	9.79	0.7	0.187
Positive control (n = 75)	118.6	143.2	267.4	1.07	82.32	94.17	61.81	6.18	2.854	3.561	2.2	22.05	11.882	14.12	22	5.5	6.703	7.145	0.9	0.252
Negative control (n = 75)	37.1	45.78	332	13.3	24.41	30.25	135.5	1.36	0.844	1.212	0.6	59.63	7.072	7.471	0.7	0.176	4.065	4.414	0.5	0.135

(ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; TBL: Total Bilirubin; G1: Group 1 (with SVR); G2: Group 2 (non-responders); BT: Before Treatment; Positive Control: HCV ELISA Positive; Negative Control: HCV ELISA negative).

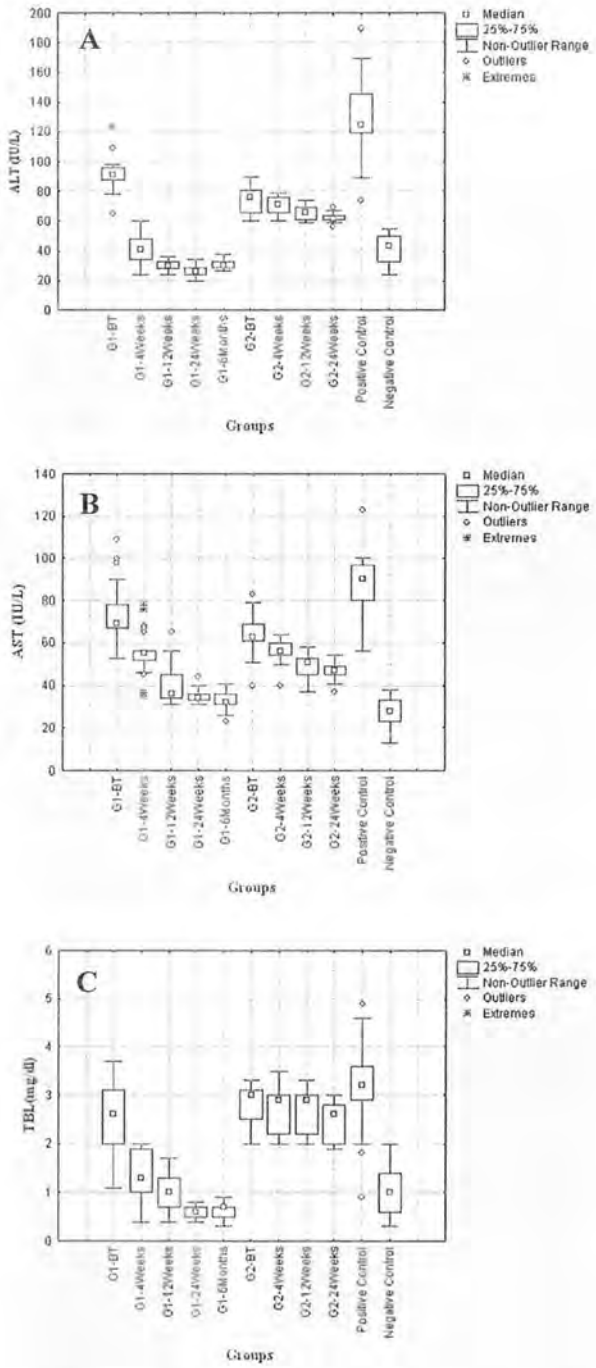

Figure 3.1.5: Variation of significant serum markers among treatment groups (A) ALT (B) AST (C) TBL for treatment and control groups.

(ALT: Alanine Aminotransferase; AST: Aspartate Aminotransferase; TBL: Total Bilirubin; G1: Group 1 (with SVR); G2: Group 2 (non-responders); BT: Before Treatment; Positive Control: HCV ELISA Positive; Negative Control: HCV ELISA negative).

Serum total protein and albumin also showed significant decrease from 13 ± 1.16 and 6 ± 0.76 to 7± 1.26 and 4.88 ± 0.59 respectively (P = 0.001, P = 0.000 and P = 0.002) as the therapy progressed. No significant difference was observed till 4th week however at 12th week the patients showed positive and consistent response towards therapy in terms of decrease in serum albumin and total protein that prolonged even when analyzed after 6 month from the end of the therapy in patients of group 1 (with SVR) (Table 3.1.2 and Figure 3.1.6). In group 2 the patients who had no effect of therapy on their viral load showed less significant drop in serum ALT (P = 0.014) and AST (P = 0.001) from 74.76 ± 8.44 IU/L and 63.88 ± 9.09 IU/L to 62.28 ± 2.77 IU/L and 47 ± 3.96 IU/L respectively. Before 12th week of treatment, no significant response was observed. Moreover in contrast to group 1 the drop in serum concentrations of ALT and AST in group 2 becomes similar to the positive control group instead of negative control group (Table 3.1.2 and Figure 3.1.5). Serum levels of albumin in non-responders got decreased significantly till 24 weeks of treatment and the values became comparable to the negative control group. Moreover serum total protein also showed significant decrease in response to therapy but the decrease is not comparable to negative control group (Table 3.1.2 and Figure 3.1.6).

3.1.3.2.1 Association of Liver Profile with HCV Viral Load

Correlation of the liver profile with the viral load was determined using statistical software Statistica version 7.0. On the basis of r and p values the association of liver profile with viral load was determined. Overall the liver profile remained directly correlated in patients who respond positively to therapy (group 1) and showed decline in their viral load with the progression of therapy. Only albumin showed strong positive correlation at 4th week of treatment that also changes to weak association till 12th week of treatment (Table 3.1.3 and Figure 3.1.7).

While in group 2 serum ALT, AST and total protein showed linear reduction with the multiplication of the viral RNA copies whereas concentration of serum TBL and albumin increased with the increase in RNA copies. The response of the group 2 is similar to the positive control group (Table 3.1.3 and Figure 3.1.8).

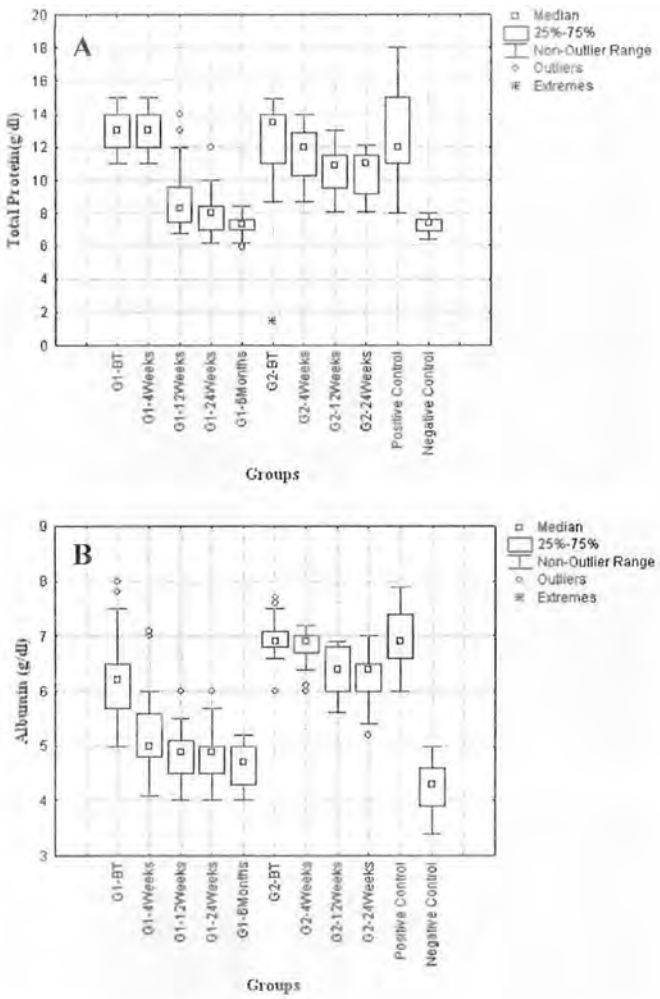

Figure 3.1.6: Comparative analysis of total protein and albumin at different treatment periods in both groups. Box plots of: (A) Total Protein (B) Albumin for treatment and control groups.

(G1: Group 1 (with SVR); G2: Group 2 (non-responders); BT: Before Treatment; Positive Control: HCV ELISA Positive; Negative Control: HCV ELISA negative).

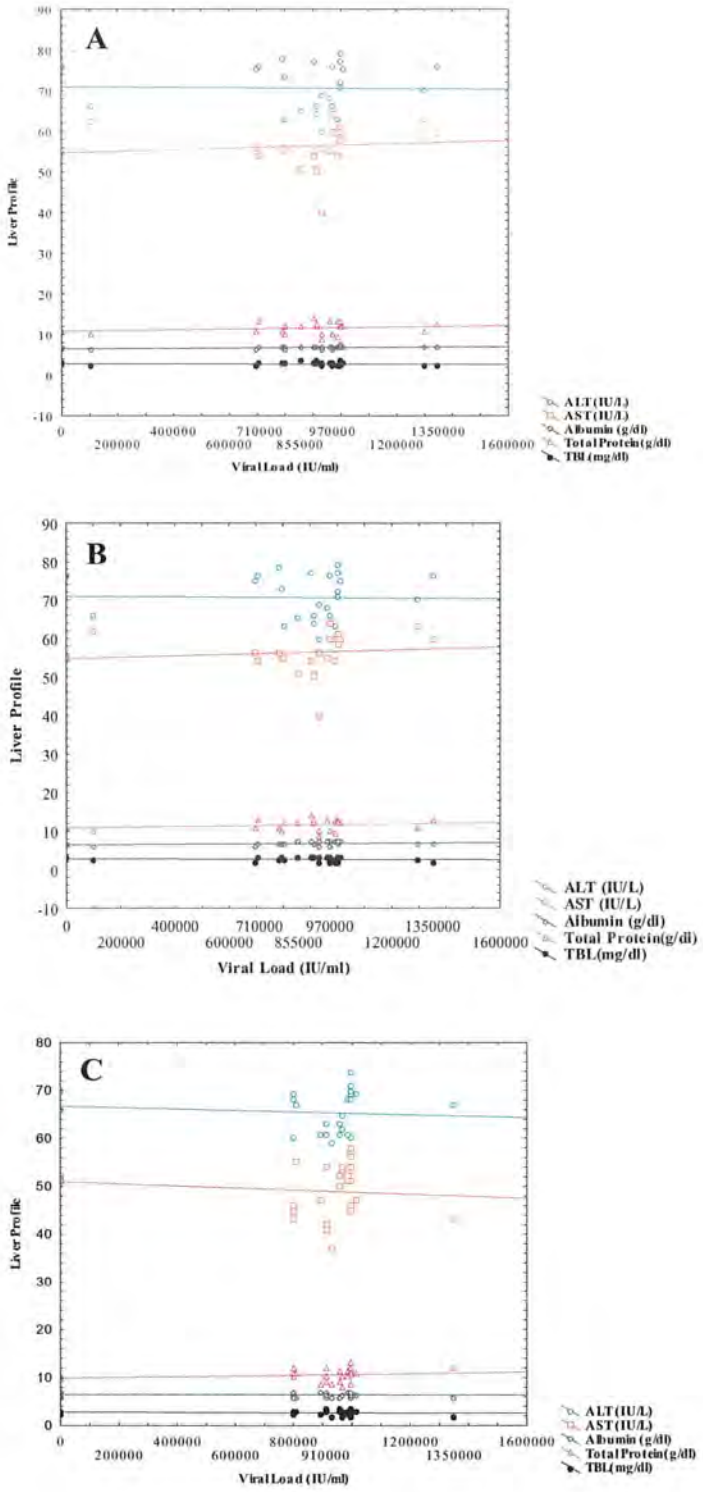

Figure 3.1.7: Correlation of liver profile with the viral load in group 1 (with SVR) at different treatment duration (A) before treatment (B) 4 weeks of treatment (C) 12 weeks of treatment.
(ALT: Alanine Amino Transferase; AST: Aspartate Amino Tranferase; TBL: Total Bilirubin)

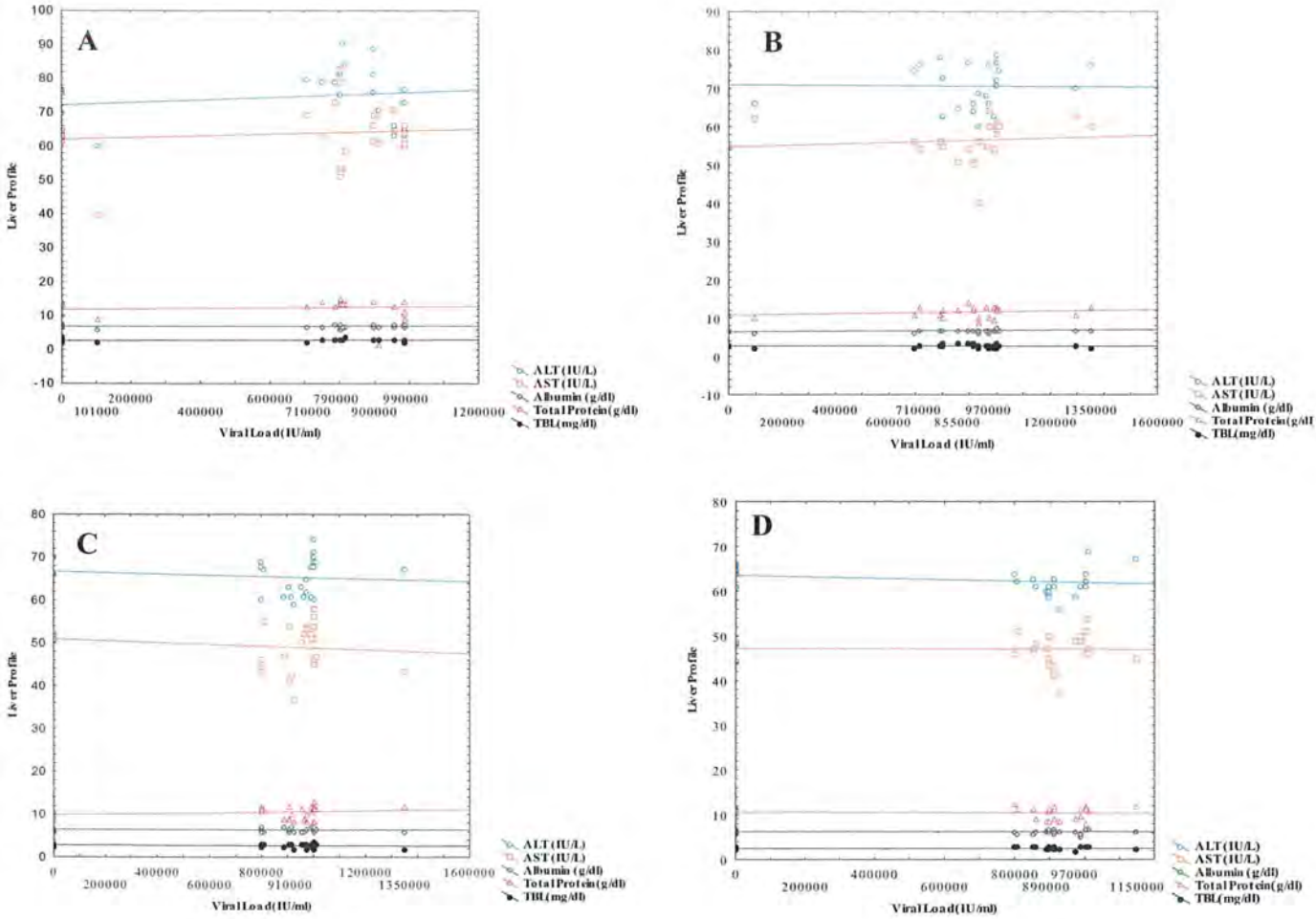

Figure 3.1.8: Correlation of liver profile with the viral load in group 2 (non-responders) at different treatment duration (A) before treatment (B) 4 weeks of treatment (C) 12 weeks of treatment (D) 24 weeks of treatment.

(ALT: Alanine Amino Transferase; AST: Aspartate Amino Tranferase; TBL: Total Bilirubin)

3.1.3.3 Comparative Analysis of Lipid Profile in All Studied Cohorts

In the present study both the treatment and control groups were analyzed for lipid profile and comparison was made among all the groups using univariate analysis by taking significance $P > 0.05$ and LSD was taken at 0.01.

Patients in group 1 (who exhibited SVR) showed significant decrease of serum LDL ($P = 0.000$), HDL ($P = 0.000$), TCOL ($P = 0.000$) and TG ($P = 0.000$) from 125.6 ± 4.76 mg/dl, 57.52 ± 3.90 mg/dl, 160 ± 23.7 mg/dl and 177.44 ± 165 mg/dl to 114.6 ± 17.72 mg/dl, 50.56 ± 6.21 mg/dl, 130.14 ± 14.29 mg/dl and 131.70 ± 13.11 mg/dl respectively. Serum LDL and TG showed response at 12^{th} week of treatment and then consistent decrease till 24^{th} week of treatment that even continued after 6 month end of therapy, while HDL and TCOL showed immediate decrease after 4 weeks of treatment that lasts till the end of therapy (Table 3.1.4; Figure 3.1.9 and 3.1.10).

In group 2 (non-responders) the patient's serum concentrations of LDL, HDL, TCOL and TG were 165.92 ± 17.90 mg/dl, 69.68 ± 4.99 mg/dl, 191.72 ± 11.8 mg/dl and 237.04 ± 24.00 mg/dl before the start of therapy. These concentrations reach to 125.6 ± 17.72 mg/dl ($P = 0.002$), 50.56 ± 6.21 mg/dl ($P = 0.003$), 179.64 ± 14.29 mg/dl ($P = 0.932$) and 197.72 ± 13.11 mg/dl ($P = 0.032$) after 24^{th} week of treatment. However, these values were not much different from positive control groups. LDL, HDL and TG showed effect at 12 weeks whereas TCOL did not show any change throughout treatment period (Table 3.1.4; Figure 3.1.9 and 3.1.10).

Hence in the present study the patients who respond to therapy (group 1) showed improvement in their lipid profile and normalization of serum concentrations of LDL, HDL, TGs, and serum total cholesterol occurred with the progression of the therapy. While the patients who failed to respond therapy (group 2) did not show any significant change in their lipid profile (Table 3.1.4; Figure 3.1.9 and 3.1.10).

Table 3.1.4: Univariate analysis of patient's lipid profile in all study cohorts.

Treatment	HDL (mg/dl)				LDL (mg/dl)				TCOL (mg/dl)				TG (mg/dl)			
	95 % Confidence Interval (CI)		Mean Square (MS)	F-Value	95 % Confidence Interval (CI)		Mean square (MS)	F-Value	95 % Confidence Interval (CI)		Mean Square (MS)	F-Value	95 % Confidence Interval (CI)		Mean Square (MS)	F-Value
	lower limit	upper limit			lower limit	upper limit			lower limit	upper limit			lower limit	upper limit		
	Group 1 (n = 376)															
Before start of treatment	78.81	89.75	52.69	58.54	134.94	158.18	2379	23.79	240.4	249.64	379	3.79	226.6	268.3	7647	76.47
4th week	63.526	69.754	52.69	58.54	135.25	144.83	2379	23.79	218.3	227.61	379	3.79	223.94	264.3	7647	76.47
12th week	54.337	58.783	87.02	9.66	121.48	124.12	30.5	0.305	156	187.24	228.75	42.87	163.77	189.8	2988	29.88
24th week	55.908	59.132	45.78	5.08	112.43	116.37	68.25	0.6825	120.4	139.57	161.1	16.11	126.83	136.1	374	3.74
6 month after the end of therapy	44.394	48.166	62.63	6.958	96.689	100.35	59.03	0.5903	121.1	141.2	177.32	17.73	123.6	137.4	834.5	8.345
	Group 2 (n = 75)															
Before start of treatment	68.171	72.949	74.93	8.325	71.273	78.24	961.5	9.614	163.68	167.123	409.88	4.099	61.134	63.43	1729	17.29
4th week	67.617	71.743	39.62	4.40	118.28	132.92	174.4	17.435	164.54	167.54	243.93	2.439	59.664	63.46	188.3	1.883
12th week	60. 54	64.66	63.27	7.03	62.3	65.6	175.1	17.506	211.15	213.509	426.98	7.269	10.969	12.13	181.2	1.812
24th week	17.906	18.974	116	12.89	19.57	20.67	942.3	9.4225	198.45	219.71	465.32	12.00	47.554	59.49	516.4	5.164
Positive control (n = 75)	78.887	89.753	51.97	20.78	168.1	178.62	487.2	4.8722	325.2	362.99	429.37	62.94	189.94	208.1	1457	14.57
Negative control (n = 75)	42.479	46.321	65	2.6	71.29	82.22	526.3	5.2632	125.7	134.68	353.5	3.535	121.77	142.2	1828	18.28

(LDL: Low Density Lipoproteins; HDL: High Density Lipoproteins; TCOL: Total Cholesterol; TGs: Triglycerides; G1: Group 1 (with SVR); G2: Group 2 (non-responders); BT: Before Treatment; Positive Control: HCV ELISA Positive; Negative Control: HCV ELISA negative).

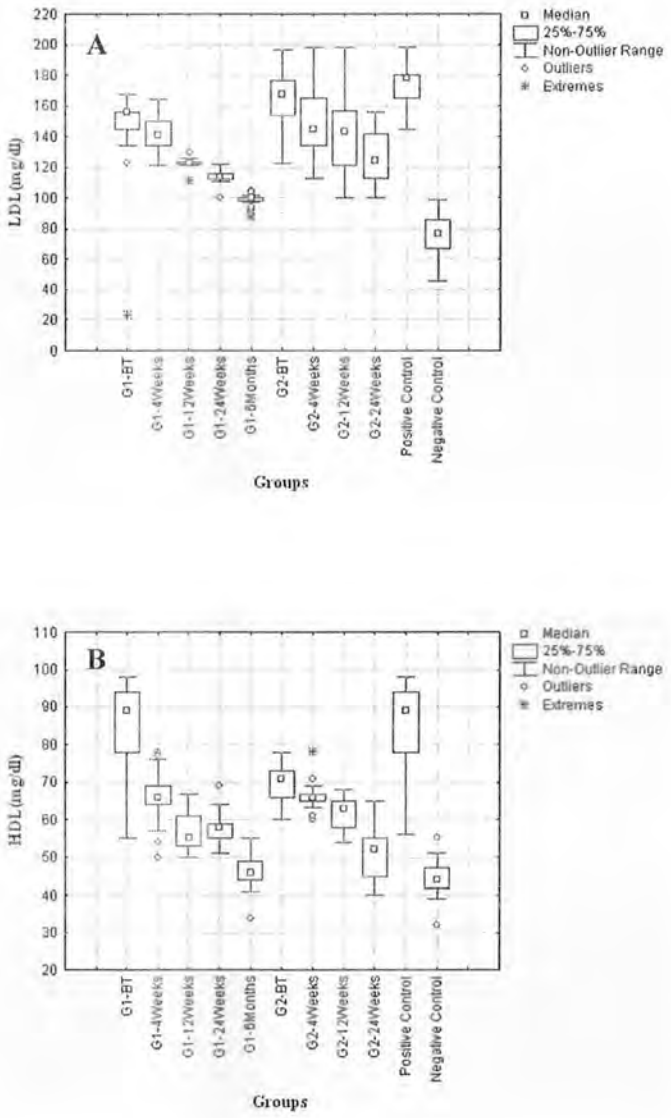

Figure 3.1.9: Comparative analysis of LDL and HDL at different treatment periods among both treatment groups along with control groups. Box plot of: (A) LDL (B) HDL for treatment and control groups.

(LDL: Low Density Lipoproteins; HDL: High Density Lipoproteins; G1: Group 1 (with SVR); G2: Group 2 (non-responders); BT: Before Treatment; Positive Control: HCV ELISA Positive; Negative Control: HCV ELISA negative).

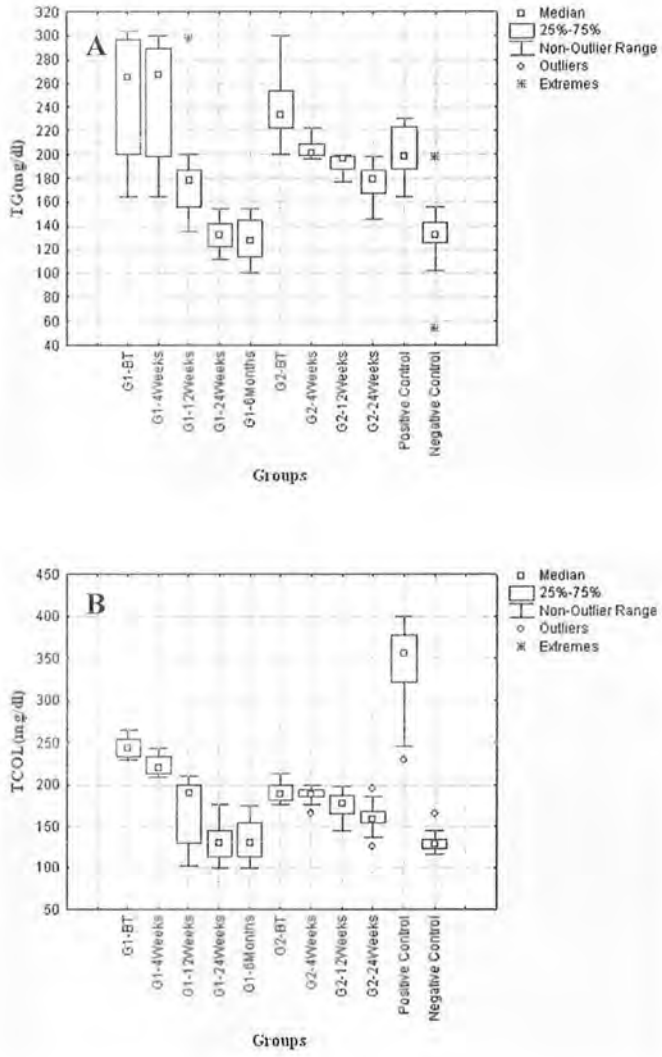

Figure 3.1.10: Comparative analysis of TG and TCOL at different treatment periods among both treatment groups along with control groups. Box plot of: (A) TG (B) TCOL for treatment and control groups.

(TG: Triglycerides; TCOL: Total Cholesterol; G1: Group 1 (with SVR); G2: Group 2 (non-responders); BT: Before Treatment; Positive Control: HCV ELISA Positive; Negative Control: HCV ELISA negative).

3.1.3.3.1 Correlation of Serum Lipid Profile with the HCV Viremia

In the present study the patients who become HCV RNA negative in response to therapy (group 1), the lipid profile (LDL, HDL, TCOL and TG) showed weak but direct correlation with the HCV viremia at 12th week of treatment though initially it remained inversely related and also non-significantly as well till 4 weeks of treatment (Table 3.1.5 and Figure 3.1.11). In non-responders (group 2) LDL exhibited weak negative correlation with the HCV viremia till even 24th week of treatment while HDL was initially positively related then a negative impact occurred at 12th week of treatment that changed to the positive again at 24 weeks of treatment. TCOL showed negative correlation till 4th week of treatment that changed to the positive one till 24 weeks of treatment. While TGs showed negative correlation till 12th week of treatment that changed to positive one at 24th week of treatment (Table 3.1.5 and Figure 3.1.12).

3.1.3.4 Comparative Analysis of Selected Blood Parameters among all the Studied Groups

Comparative analysis of selected blood parameters among the studied groups was also carried out. The patients with SVR (group 1) had mean values of Hb, PTT, and PLT, 19 ± 2.98 mg/dl, 59 ± 7.77 seconds and 73 ± 8.64 ($\times 10^3/mm^3$) respectively before the start of therapy that showed significant change to 14.24 ± 1.64 mg/dl (P = 0.000), 35 ± 4.89 seconds (P = 0.000) and 223 ± 30.09 ($\times 10^3/mm^3$) (P = 0.000) till the 24th week of treatment. All the three parameters showed progressive change as the therapy proceeded (Table 3.1.6 and Figure 3.1.13). In group 2 (non-responders) patients showed the serum concentration of Hb, PTT and PLT as 20.36 ± 1.35 mg/dl, 53 ± 14.45 seconds and 93.44 ± 6.54 ($\times 10^3/mm^3$) before the start of therapy that reached to 19.08 ± 1.53 mg/dl, 42.96 ± 5.02 seconds and 135.04 ± 21.32 ($\times 10^3/mm^3$) that is less significantly changed with P values of 0.984, 0.765 and 0.986 respectively when compared with negative control group. Hb and PTT did not show change after 4 weeks of treatment that persists (Table 3.1.6 and Figure 3.1.13).

Table 3.1.5: Correlation of serum lipid profile with HCV viral load.

Treatments	LDL (mg/dl)		HDL (mg/dl)		TCOL (mg/dl)		TG (mg/dl)	
	r	p	r	p	r	p	r	p
Group 1(n = 376)								
Before start of treatment	-0.2434	0.2411	-0.161	0.4421	-0.048	0.82	-0.0034	0.9872
After 4 weeks	-0.2434	0.2411	-0.161	0.4421	-0.048	0.82	-0.0034	0.9872
After 12 weeks	0.1453	.4884	0.3315	0.1055	0.3171	0.1225	-0.1565	0.4551
Group 2 (n = 75)								
Before start of treatment	-0.1476	0.4814	0.1226	0.5593	-0.23	0.2697	-0.1419	0.4986
After 4 weeks	-0.1476	0.4814	0.1226	0.5593	-0.23	0.2697	-0.1419	0.4986
After 12 weeks	-0.1595	0.4464	-0.196	0.3467	0.203	0.3304	-0.1025	0.6259
After 24 weeks	-0.2107	0.3121	0.0476	0.8211	-8904	0.997	0.1128	0.5913

(LDL: Low Density Lipoproteins; HDL: High Density Lipoproteins; TCOL: Total Cholesterol; TGs: Triglycerides; G1: Group 1 (with SVR); G2: Group 2 (non-responders); BT: Before Treatment; r: Correlation Coefficient; p: Statistically Significant).

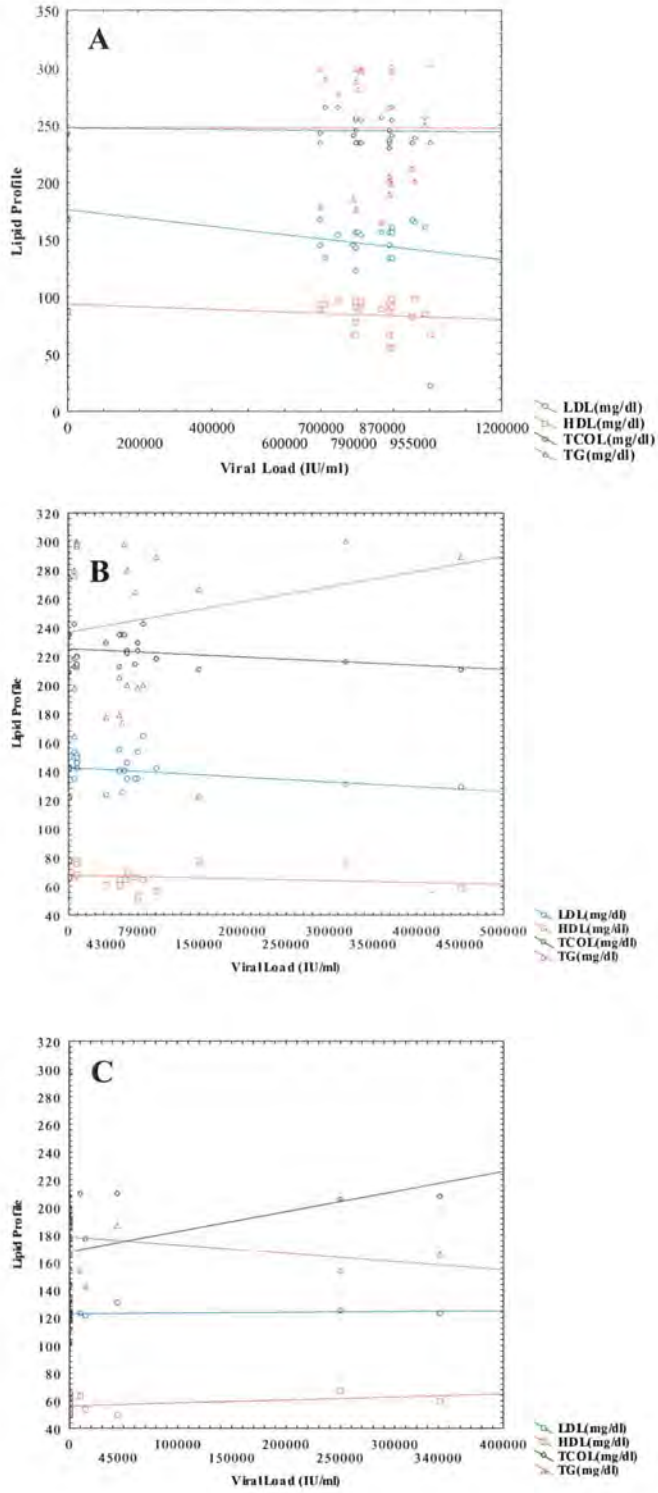

Figure 3.1.11: Correlation of lipid profile with viral load in group 1 (with SVR) at different treatment periods (A) before treatment (B) 4 weeks (C) 12 weeks.
(LDL: Low Density Lipoproteins; HDL: High Density Lipoproteins; TCOL: Total Cholesterol; TGs: Triglycerides)

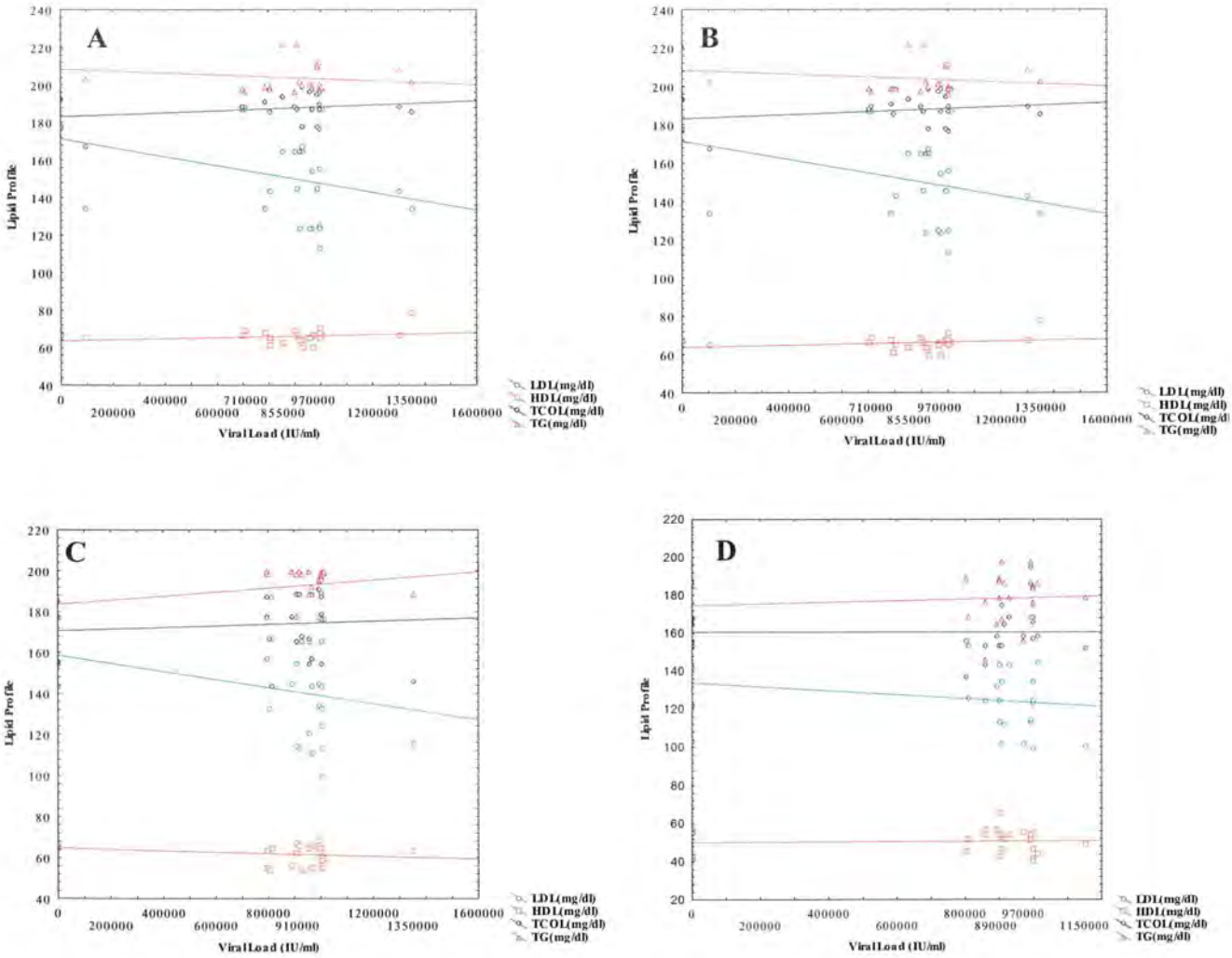

Figure 3.1.12: Correlation of lipid profile with viral load in group 2 (non-responders) at different treatment periods (A) before treatment (B) 4 weeks (C) 12 weeks (D) 24 weeks.

(LDL: Low Density Lipoproteins; HDL: High Density Lipoproteins; TCOL: Total Cholesterol; TGs: Triglycerides)

Table 3.1.6: Univariate analysis of selected blood parameters of all the treatment groups.

Treatment	PTT (seconds)				Hb (g/dl)				PLT (×10³/mm³)			
	95% Confidence interval (CI)		Mean Square (MS)	F-Value	95% Confidence interval (CI)		Mean Square (MS)	F-Value	95% Confidence interval (CI)		Mean Square (MS)	F-Value
	lower limit	upper limit			lower limit	upper limit			lower limit	upper limit		
Group 1 (n = 376)												
Before start of treatment	55.392	61.808	181.25	7.25	18.167	20.633	26.75	1.07	69.72	76.608	224.12	2.2412
After 4 weeks	49.081	53.479	181.25	7.25	16.017	16.943	26.75	1.07	96.857	101.7	224.12	2.2412
After 12 weeks	36.607	40.353	61.78	2.4712	14.432	15.808	8.33	0.333	130	139.84	425.98	4.259
After 24 weeks	32.981	37.019	71.75	2.87	13.574	14.906	7.82	0.3128	210.58	235.42	271.75	27.1775
6 months	33.985	36.815	35.25	1.41	12.69	13.87	6.13	0.2452	262.24	284.16	215.75	21.1575
Group 2 (n = 75)												
Before start of treatment	2.5259	2.9301	626.78	25.071	26.13	29.77	5.22	0.208	79.91	87.69	125.02	1.25
After 4 weeks	227.13	246.95	72.57	2.902	11.81	28.31	5.33	0.2132	200.61	207.15	476.5	4.765
After 12 weeks	210.63	227.05	62.22	2.488	23.51	25.67	5.02	0.2008	256.02	284.62	466	4.66
After 24 weeks	121.46	131.74	78.12	3.1248	10.2	11.6	6.98	0.279	126.24	143.84	133.37	13.6337
Positive control (n = 75)	56.248	72.312	1135.9	11.359	19.161	20.359	6.32	0.252	90.912	116.93	299.23	29.7923
Negative control (n = 75)	37.566	41.224	56.03	0.56	14.873	16.567	12.63	0.505	286.55	343.77	14407.92	144.079

(Hb: Hemoglobin; PTT: Prothrombin Time; PLT: Platelet Count; G1: group 1 (with SVR); G2: group 2 (non-responders); BT: before treatment; Positive Control: HCV ELISA Positive; Negative Control: HCV ELISA Negative).

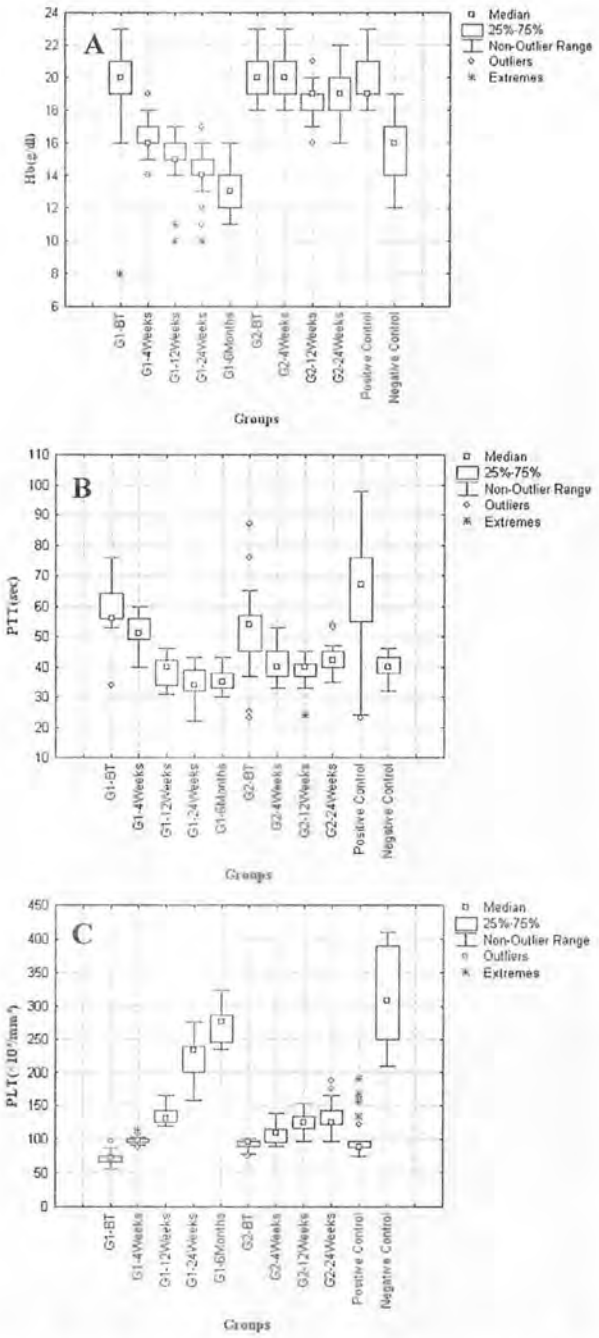

Figure 3.1.13: Comparative analysis of selected blood parameters at different treatment periods among both treatment groups along with control groups. Box plot of: (A) Hb (B) PTT (C) PLT for treatment and control groups.

(Hb: Hemoglobin; PTT: Prothrombin Time; PLT: Platelet Count; G1: group 1 (with SVR); G2: group 2 (non-responders); BT: before treatment; Positive Control: HCV ELISA Positive; Negative Control: HCV ELISA Negative).

3.1.3.4.1 Correlation of HCV Viremia with Hematological Indices

In patients who become HCV RNA negative after receiving the therapy (group 1), the hemoglobin level showed weak plus negative correlation till 4th week of treatment that become strongly negative correlation till the 12th week of treatment. Prothrombin time remain negatively correlated with the HCV viremia and become positively and non-significantly related with HCV viremia. Moreover, PLT remain negatively correlated with the therapy intake during the whole period of treatment (Table 3.1.7 and Figure 3.1.14). In group 2, Hb levels remained non-significantly plus positively correlated till 24th week with some variation at 12th week of treatment. PTT time remain negatively correlated till 4th week of treatment while the response become positively related till 24th week of treatment. PLT remain negatively correlated throughout the whole period of treatment (Table 3.1.17 and Figure 3.1.15).

3.2 MOLECULAR CHARACTERIZATION OF HCV

To further accomplish the objectives of the study, besides the host factors, viral factors by themselves were also under consideration. For genetic characterization and molecular analysis only two groups, group 1 (with SVR) and group 2 (non-responders) who were on therapy were processed and compared with each other. As the total number of patients in group 2 was 75 so that from group 1 out of 376 patients, 75 patients of same age group to patients in group 2 were randomly picked for comparison of the genetic analysis of two treatment groups.

3.2.1 Study Design

To find the molecular basis of HCV response to antiviral therapy, group 1 (with SVR) and group 2 (non-responders) were further processed for the molecular characterization. Serums samples were collected from patients of both treatment groups for RNA extraction and were further amplified after reverse transcribing them for concerned genomic region to analyze for genotyping.

Table 3.1.7: Correlation of blood parameters with viral load.

Treatments	Hb (g/dl)		PTT (seconds)		PLT ($\times 10^3/mm^3$)	
	r	p	r	p	r	p
Group 1 (n = 376)						
Before start of treatment	-0.0485	0.818	-0.2328	0.2628	-0.0447	0.832
After 4 weeks	-0.3243	0.1138	-0.3654	0.0725	-0.1689	0.4196
After 12 weeks	-0.5074	0.0096	0.0286	0.8922	-0.2887	0.1677
Group 2 (n = 75)						
Before the start of treatment	0.1484	0.4789	-0.0138	0.948	-0.2369	0.2541
After 4 weeks	0.1484	0.47789	0.0138	0.948	-0.2369	0.2541
After 12 weeks	-0.1836	0.3798	0.1239	0.5551	-0.2564	0.2161
After 24 weeks	0.0507	0.81	0.2749	0.1835	-0.22772	0.2748

(Hb: Hemoglobin; PTT: Prothrombin Time; PLT: Platelet Count; G1: group 1 (with SVR); G2: group 2 (non-responders); r: Correlation Coefficient; p: Statistically Significant).

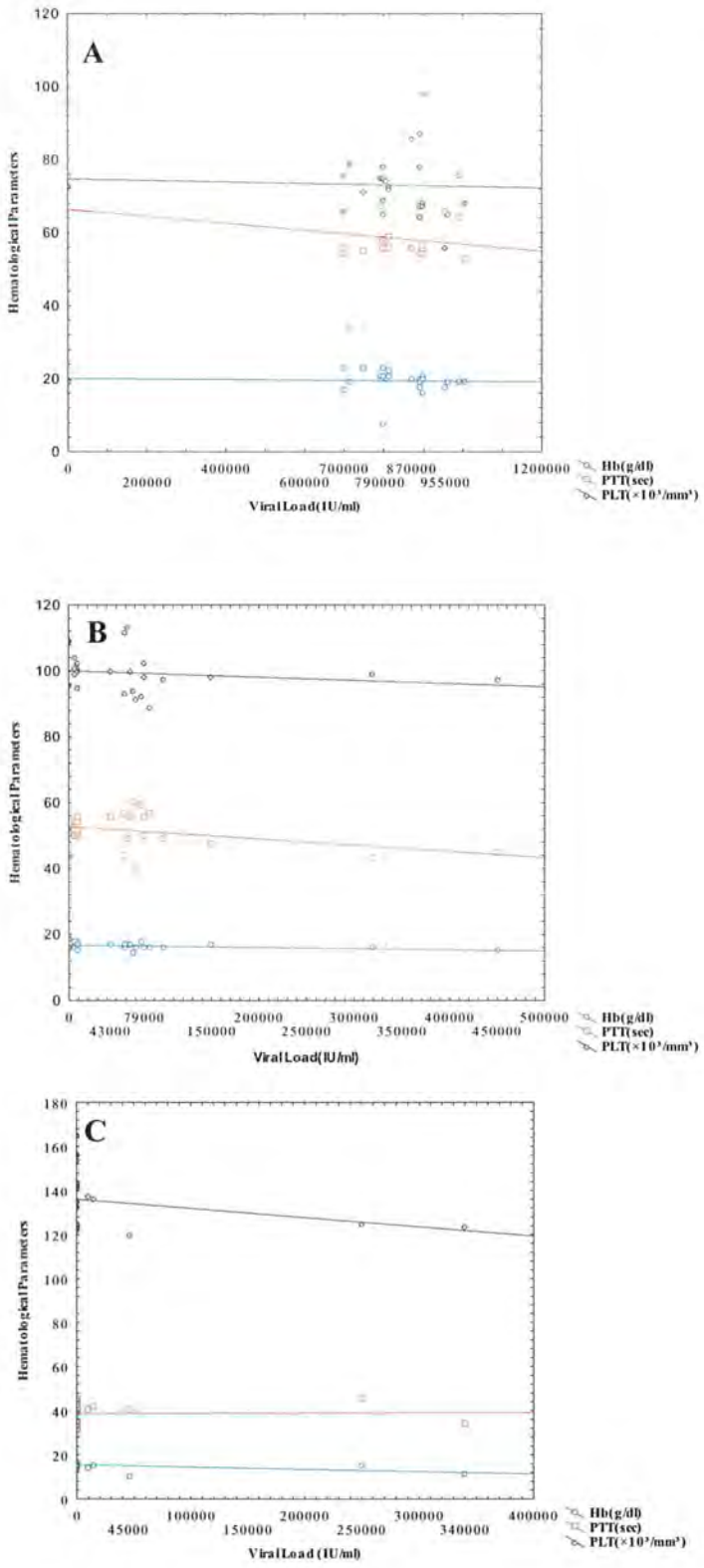

Figure 3.1.14: Correlation of Hb, PLT and PTT with viral load in group 1 (with SVR) at different treatment periods (A) before treatment (B) 4 weeks (C) 12 weeks.
(Hb: Hemoglobin; PTT: Prothrombin Time; PLT: Platelet Count)

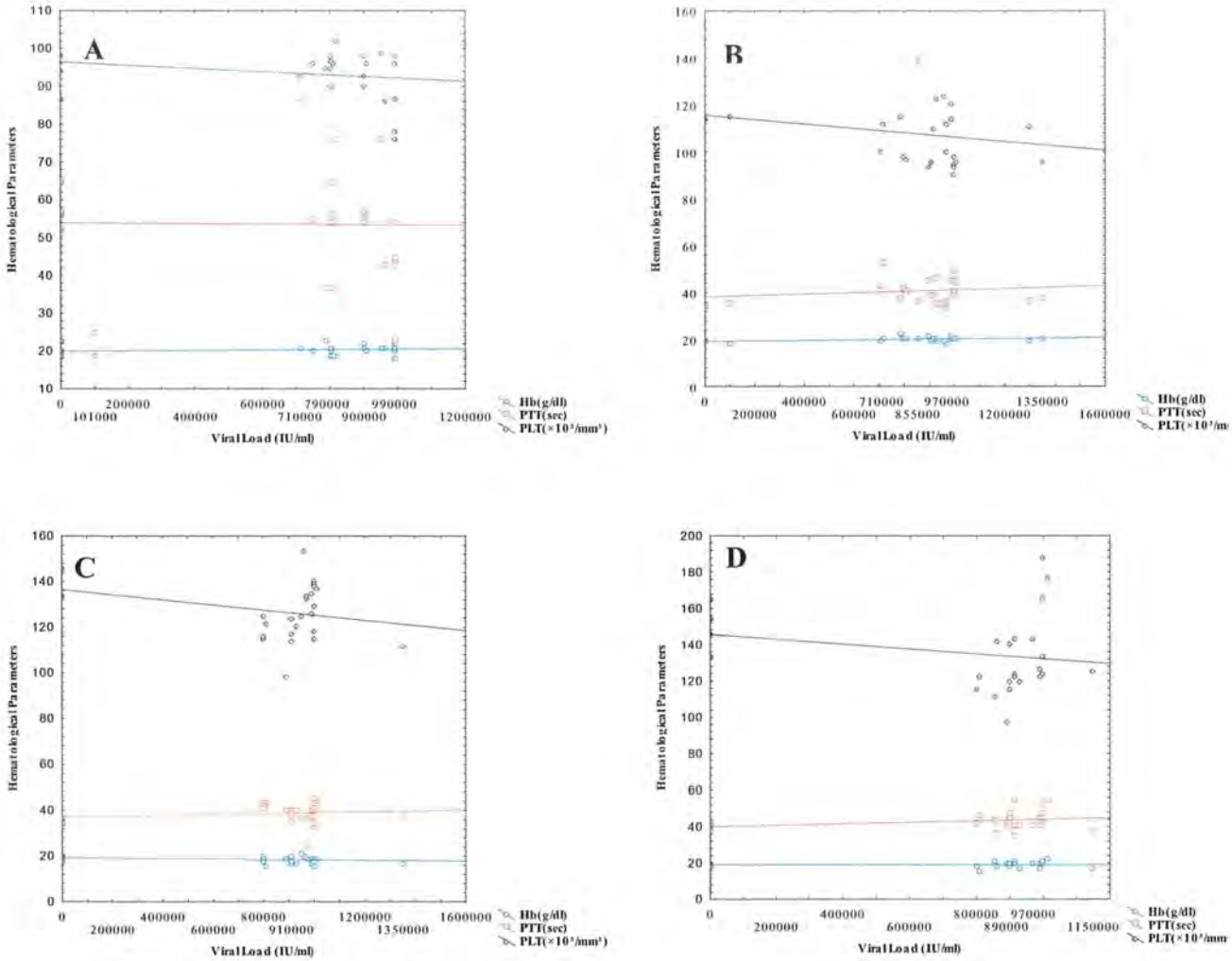

Figure 3.1.15: Correlation of selected blood parameters with viral load in group 2 (non- responders) at different treatment periods (A) before treatment (B) 4 weeks (C) 12 weeks (D) 24 weeks
(Hb: Hemoglobin; PTT: Prothrombin Time; PLT: Platelet Count)

3.2.3 RT-PCR, Nested PCR and Agarose Gel Electrophoresis

HCV being single stranded sense virus is unable to be taken for molecular amplification without making a cDNA. The cDNA synthesis was done using external forward primer under controlled conditions for each of the three genomic regions, for all the samples of both the groups and stored at -80 °C to prevent its degradation. The cDNA was further processed by two independent rounds of PCR. The products of nested PCR were run on 1.5 % agarose, made in 0.5 X TAE buffer and the gel was stained using ethidium bromide. Positive and negative controls were used whenever the amplifications were performed, in order to avoid false positive and false negative results.

Electrophoresis revealed a 256 bp product for 5' UTR (Figure 3.2.1), 695 bp product for NS5B (Figure 3.2.2) and 404 bp product for core (Figure 3.2.3) as per expectation. Huge quantities of the HCV RNA were amplified for further analysis.

3.2.4 Sequencing of Amplified Genomic Regions

During the present study the amplified products for all the samples of both groups were sequenced for three selected genomic regions. For sequencing reaction high class DNA was used and high quality sequencing was observed (Figure 3.2.4).

3.2.5 Blast Analysis

Blast analysis was performed for all the sequences. The objective was to resolve the genotypes of the sequenced samples. The results of blast analysis provided high sequence similarity scores between the analyzed sequences and already reported sequences of 5' UTR, Core and NS5B. Percentage similarity of sequenced sample with already reported sequences from blast is mentioned in table 3.2.1.

3.2.5.1 Genotype 3

Blast analysis revealed the presence of genotype 3a and 3b in both treatment groups (1 and 2). Out of 75 patients in group 1 (with SVR) 36 patients possess genotype 3b while 20 patients showed genotype 3a (Table 3.2.1 and Figure 3.2.5), whereas among non-responders (group 2) only 2 patients showed the presence of genotype 3b and 10 patients revealed the genotype 3a and 3 patients were presented with genotype 3c (Figure 3.2.6 and Table 3.2.2).

Figure 3.2.1: RT-PCR amplification of 5′ UTR region from studied HCV samples. **Lane 1**: 50 plus bp ladder (Fermentas), **lane 2**: positive control, **lane 3**: negative control and **lane: 4-10** represents HCV positive samples.

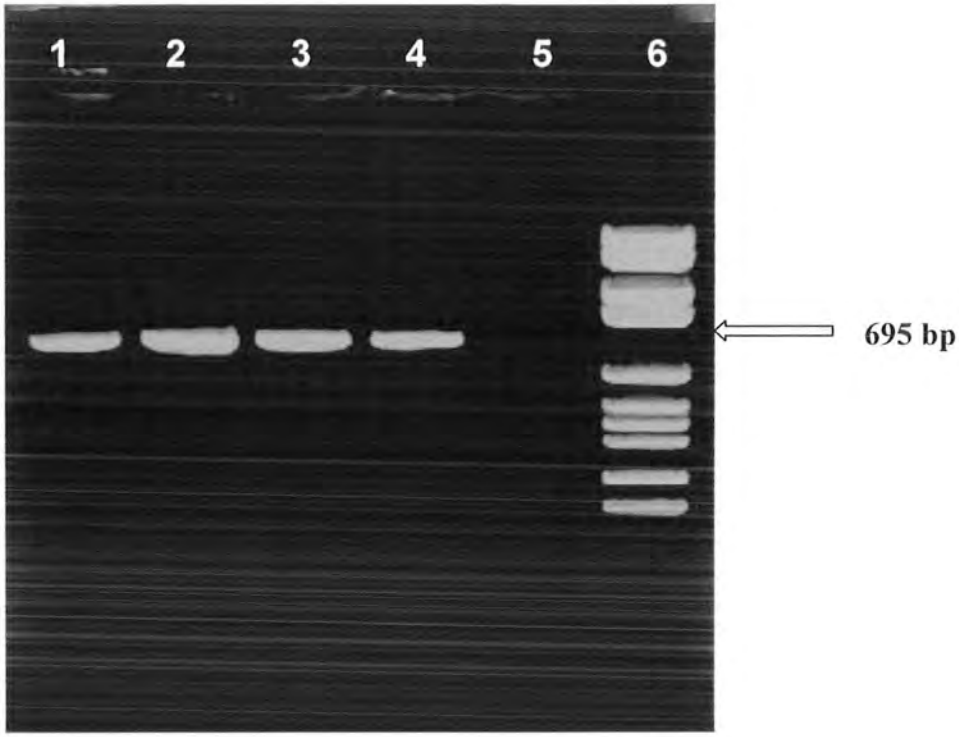

Figure 3.2.2: RT-PCR amplification of NS5B region from HCV samples. **Lane 1-3** representative HCV positive samples, **lane 4**: positive control, **lane 5**: negative control and **lane 6**: 100 bp ladder (Fermentas).

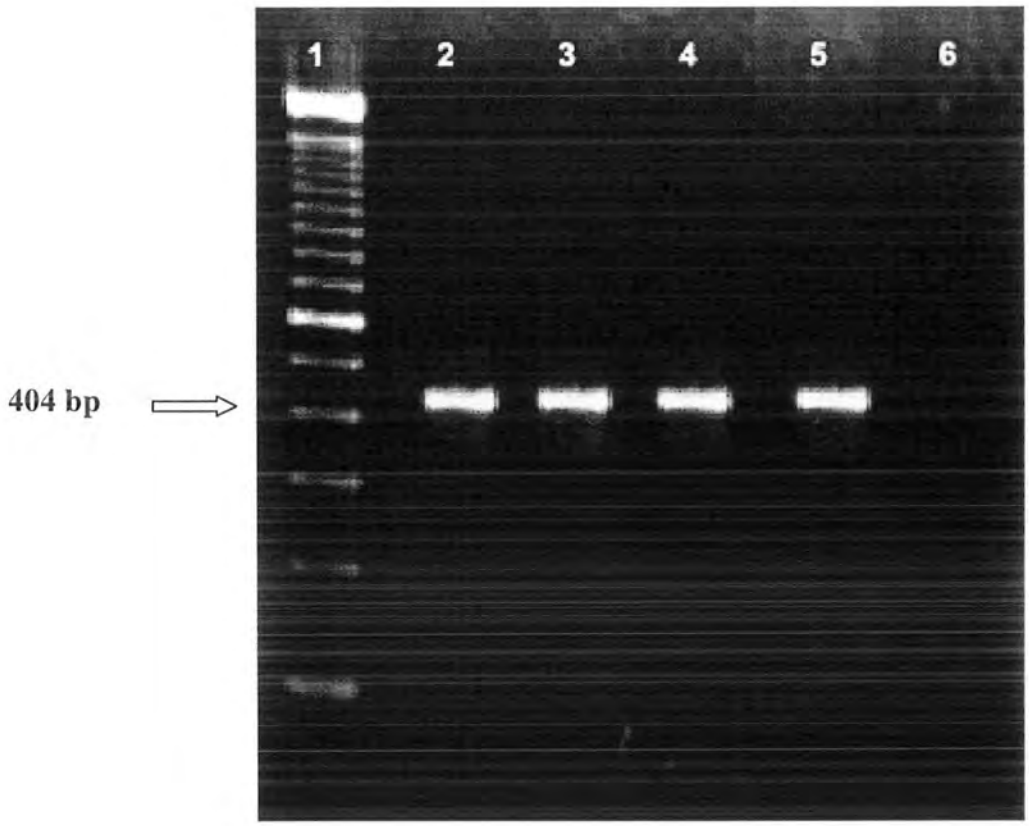

Figure 3.2.3: RT-PCR amplification of core region from HCV samples. **Lane 1**: 100 bp ladder (Fermentas), **lane 2-4**: HCV positive sample, **lane 5** positive control and **lane 6:** negative control.

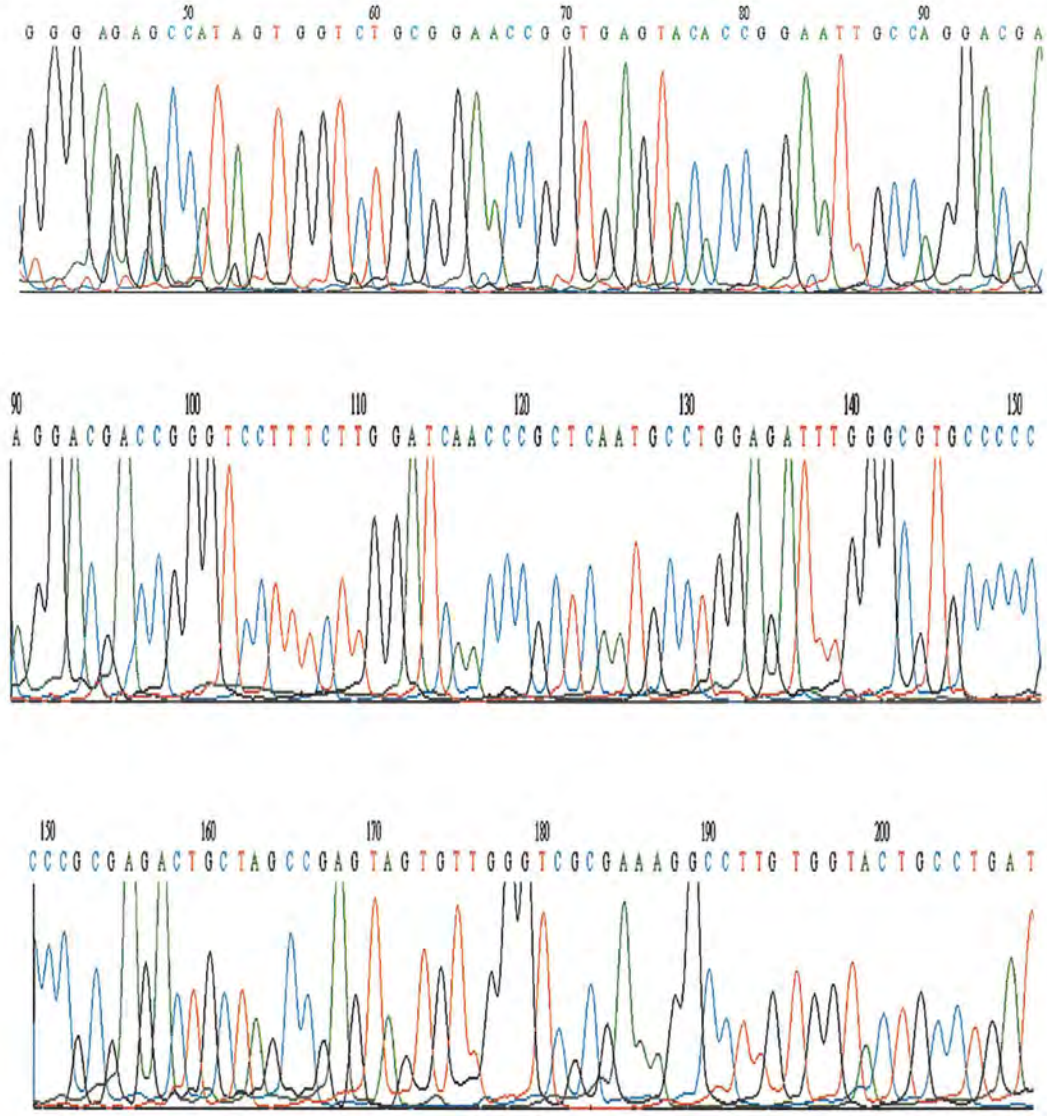

Figure 3.2.4: Results of sequencing in the form of sharp peaks.

Number of patients exhibiting genotype 3 (a and b) is more in group 1 (SVR) as compared to group 2 (non-responders) describing the positive response of genotype 3 to antiviral therapy.

3.2.5.2 Genotype 1

Present study showed the presence of genotype 1 in both treatment groups. In group 1, 10 patients exhibited the genotype 1b (Figure 3.2.5 and Table 3.2.1) while in group 2, 8 patients presented with genotype 1b and 5 with genotype 1a and 4 with 1c (Figure 3.2.6 and Table 3.2.2). Overall in our study, genotype 1 (a and b) is more prevalent in non-responders as compared to the patients who respond to the therapy.

3.2.5.3 Genotype 4

Only 9 patients in group 1 (with SVR) showed similarity with genotype 4a (Figure 3.2.5 and Table 3.2.1) while 13 patients from group 2 (non-responders) exhibited resemblance with this genotype 4 (6 patients with genotype 4a, 4 patients with genotype 4b and 3 patient with genotype 4c) (Figure 3.2.6 and Table 3.2.2).

3.2.5.4 Genotype 5

There is no observation about the presence of genotype 5 in group 1 (with SVR) (Figure 3.2.5 and Table 3.2.1) while 8 patients showed the presence of genotype 5 (4 patients with genotype 5a and 4 patients with genotype 5b) in group 2 (non-responders) in the present study (Figure 3.2.6 and Table 3.2.2).

3.2.5.5 Genotype 2

Patients who respond positively to therapy did not present the prevalence of genotype 2 in group 1 whereas in group 2, 9 patients were presented with genotype 2 (5 patients with genotype 2b while genotype 2a and 2c were presented by 2, 2 patients each) (Figure 3.2.5 and Table 3.2.2).

3.2.5.6 Genotype 6

Patients exhibited SVR (group 1) did not show the presence of genotype 6 (Figure 3.2.5 and Table 3.2.1) while 8 patients in group 2 showed the presence of genotype 6 (5 patients with genotype 6a while 6q, 6v and 6e, one each) (Figure 3.2.6 and Table 3.2.2). Genotype 6q and 6v have been reported for the first time from Pakistan in this study.

Table 3.2.1: Blast analysis representing highest percentage similarities with already reported sequences and the resolved genotypes in group 1 (with SVR).

Sequence ID	Reference Accession for Core	% Similarity	Reference Accession for 5'UTR	% Similarity	Reference Accession for NS5B	% Similarity	Expected Genotypes
Pak-01-10 (G-1)	AY231584	91	AJ621218	89	D49374	99	3b
Pak-02-10 (G-1)	AY231585	98	AJ621231	88	JQ303541	95	3b
Pak-03-10 (G-1)	AY231586	93	D37838	87	JQ303517	96	3b
Pak-04-10 (G-1)	AY231587	99	D37839	90	JQ303515	88	3b
Pak-05-10 (G-1)	AY231589	95	D37840	91	JQ318179	86	3b
Pak-06-10 (G-1)	AY231588	96	D49374	97	JQ303511	98	3b
Pak-07-10 (G-1)	AY231590	88	AJ621218	98	JQ318179	99	3b
Pak-08-10 (G-1)	D11443	86	AJ621231	93	JQ318057	95	3b
Pak-09-10 (G-1)	D16616	98	AY231586	99	JF721321	96	3b
Pak-10-10 (G-1)	D37839	99	D16616	90	JF721322	91	3b
Pak-11-10 (G-1)	D37840	96	D37838	98	JF721320	97	3b
Pak-12-10 (G-1)	D49374	94	DQ859943	97	JQ318427	98	3b
Pak-13-10 (G-1)	DQ859943	96	AJ621220	99	JQ318363	93	3b
Pak-14-10 (G-1)	HM042021	98	AJ621222	89	JQ318363	99	3b
Pak-15-10 (G-1)	HM042022	97	D37840	87	JN572973	91	3b
Pak-16-10 (G-1)	D37838	96	HM042022	88	JN572963	98	3b
Pak-17-10 (G-1)	D37839	98	D49374	86	HQ229582	97	3b
Pak-18-10 (G-1)	D37840	97	AM502650	89	HQ229516	96	3b
Pak-19-10 (G-1)	D49374	99	AY31140	90	HQ229574	98	3b
Pak-20-10 (G-1)	DQ859943	90	D37840	90	HQ229483.	97	3b
Pak-21-10 (G-1)	HM042022	89	AY311401	91	HQ229491	98	3b
Pak-22-10 (G-1)	HM042024	88	AY311400	98	HQ229445	96	3b
Pak-23-10 (G-1)	AY231584	90	EF173939	90	HQ229423	97	3b
Pak-24-10 (G-1)	AY231586	96	JN65662	85	HQ229357	98	3b
Pak-25-10 (G-1)	D11443	98	AB444578	82	HM009305	91	3b

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Pak-26-10 (G-1)	AY11443	98	AB444578	82	HM009305	91	3b
Pak-27-10 (G-1)	AB092965	97	Q413428	90	HQ229323	97	3b
Pak-28-10 (G-1)	D16616	99	JQ303586	91	HM009303	98	3b
Pak-29-10 (G-1)	D37838	96	JQ303582	94	HM009304	93	3b
Pak-30-10 (G-1)	DQ859943	90	JQ303580	87	HM009306	99	3b
Pak-31-10 (G-1)	FR872815	96	JQ303574	98	HQ318872	91	3b
Pak-32-10 (G-1)	AB061929	98	JQ303581	80	HQ318870	97	3b
Pak-33-10 (G-1)	JQ888232	97	AM502650	91	JQ303572	98	3b
Pak-34-10 (G-1)	JQ888226	99	JQ303586	86	JQ303573	97	3b
Pak-35-10 (G-1)	JQ888233	96	D49374	97	JQ303579	96	3b
Pak-36-10 (G-1)	JQ668325.	90	AB444578	87	JQ303575	98	3b
Pak-37-10 (G-1)	JQ668323	89	D37840	89	JQ303575	97	3a
Pak-38-10 (G-1)	AB092965	96	AF046866	90	AF046866	98	3a
Pak-39-10 (G-1)	AF046866	98	AJ621215	91	D17763	97	3a
Pak-40-10 (G-1)	AM263063	97	AJ621216	90	D28917	91	3a
Pak-41-10 (G-1)	AM263063	99	AJ621217	91	DQ437509	97	3a
Pak-42-10 (G-1)	AM263066	96	AJ621219	94	GQ275355	98	3a
Pak-43-10 (G-1)	AM263067	90	AJ621220	87	GQ356206	93	3a
Pak-44-10 (G-1)	AM263069	89	AJ621222	98	GU814263	99	3a
Pak-45-10 (G-1)	AM263070	87	AJ621223	80	HQ639941	91	3a
Pak-46-10 (G-1)	AM263071	98	AJ621224	91	HQ639942	97	3a
Pak-47-10 (G-1)	AM263072	96	AJ621225	90	HQ738645	90	3a
Pak-48-10 (G-1)	AM263073	98	AJ621226	91	HQ912953	98	3a
Pak-49-10 (G-1)	AM263074	97	AJ621227	92	JN714194	97	3a
Pak-50-10 (G-1)	AM263075	99	AJ621228	91	JQ717254	99	3a
Pak-51-10 (G-1)	AM263076	96	AJ621229	90	JQ717255	90	3a

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Pak-52-10 (G-1)	AM263077	90	AJ621230	92	JQ717256	91	3a
Pak-53-10 (G-1)	AM263078	89	AJ621232	91	JQ717257	97	3a
Pak-54-10 (G-1)	AM263079	96	AJ621233	90	JQ717258	98	3a
Pak-55-10 (G-1)	AM263080	98	AJ621234	92	JQ717259	93	3a
Pak-56-10 (G-1)	AM263100	97	AJ621236	93	JQ717260	99	3a
Pak-57-10 (G-1)	AM263101	96	D17763	86	NC_009824	91	1b
Pak-58-10 (G-1)	AM263104	98	AB016785	94	AB016785	97	1b
Pak-59-10 (G-1)	AM263107	97	AB049088	89	HCVT050	98	1b
Pak-60-10 (G-1)	AM263108	99	AB049094	87	HCVT094	90	1b
Pak-61-10 (G-1)	AM263109	96	AB049099	99	HCVT109	89	1b
Pak-62-10 (G-1)	AM263110	90	AB080299	90	HCVT140	88	1b
Pak-63-10 (G-1)	AM263114	89	AB191333	98	HCVT142	87	1b
Pak-64-10 (G-1)	AM263128	87	D14484	88	HCVT145	89	1b
Pak-65-10 (G-1)	AM263133	98	D85516	94	HCVT150	90	1b
Pak-66-10 (G-1)	AM263135	99	D13558	82	HCVT161	93	1b
Pak-67-10 (G-1)	AM263136	96	AY460204	87	HCVT169	97	4a
Pak-68-10 (G-1)	AF308576	98	D45193	87	DQ418782	92	4a
Pak-69-10 (G-1)	D45193	97	DQ988075	93	DQ418783	90	4a
Pak-70-10 (G-1)	DQ418782	99	DQ988076	85	DQ418784	98	4a
Pak-71-10 (G-1)	DQ418783	96	DQ988077	91	DQ418784	90	4a
Pak-72-10 (G-1)	DQ418784	90	DQ988078	89	DQ516084	91	4a
Pak-73-10 (G-1)	DQ516084	87	GU814265	86	NC_009825	93	4a
Pak-74-10 (G-1)	DQ988073	98	NC_009825	87	Y11604	94	4a
Pak-75-10 (G-1)	DQ988074	99	DQ988075	89	DQ418789	97	4a

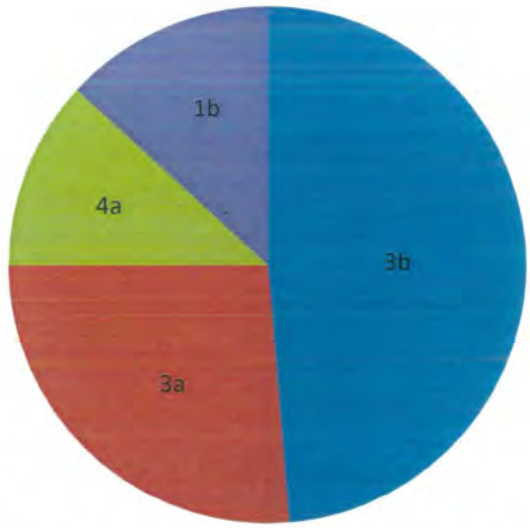

Figure 3.2.5: Frequency distribution of HCV genotypes in group 1 (with SVR).

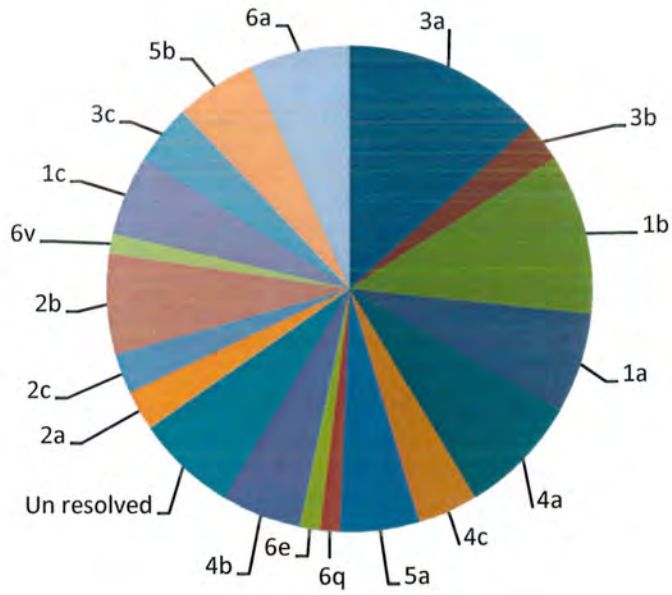

Figure 3.2.6: Frequency distribution of HCV genotypes in group 2 (non-responders).

Table 3.2.2: Blast analysis representing highest percentage similarities with already reported sequences and the resolved genotypes in group 2 (non-responders).

Sample ID	Reference Accession for UTR	% similarity	Reference Accession for Core	% similarity	Reference Accession for NS5B	% similarity	Genotype
Pak-01-10 (G-2)	AJ621215	97	AF046866	94	AF046866	93	3a
Pak-02-10 (G-2)	AJ621216	96	AY231591	94	D17763	91	3a
Pak-03-10 (G-2)	AJ621217	95	AY231592	95	D28917	90	3a
Pak-04-10 (G-2)	AJ621219	94	AY231593	98	DQ437509	98	3a
Pak-05-10 (G-2)	AJ621220	90	AY231594	93	GQ275355	89	3a
Pak-06-10 (G-2)	AJ621222	88	AY231595	92	GQ356206	88	3a
Pak-07-10 (G-2)	AJ621218	97	D37838	91	D49374	89	3b
Pak-08-10 (G-2)	AJ621231	96	D37840	90	D49374	90	3b
Pak-09-10 (G-2)	AB016785	97	AB249644	92	M62321	98	1b
Pak-10-10 (G-2)	HCVT094	99	AB191333	93	AB001390	91	1b
Pak-11-10 (G-2)	AB049094	99	EU781746	94	AB001391	90	1b
Pak-12-10 (G-2)	AB049099	90	AB435162	92	AB001392	98	1b
Pak-13-10 (G-2)	AB080299	92	AB429050	90	AB001393	93	1b
Pak-14-10 (G-2)	AB119282	92	AB191333	92	AB001399	92	1b
Pak-15-10 (G-2)	AB191333	91	AB249644	91	D28917	93	1b
Pak-16-10 (G-2)	AB520610	91	AF011752	92	AB520610	94	1a
Pak-17-10 (G-2)	AF009606	90	EF621489	98	AY885238	80	1a
Pak-18-10 (G-2)	AF011751	89	EU781746	92	NC_004102	89	1a
Pak-19-10 (G-2)	D10749	86	NC_004102	93	FJ477239	98	1a
Pak-20-10 (G-2)	DQ418784	93	DQ418784	90	AB001392	97	Unresolved
Pak-21-10 (G-2)	NC_004102	92	M62321	89	EU781746	87	1a
Pak-22-10 (G-2)	DQ418782	91	DQ418783	88	AB520610	89	4a
Pak-23-10 (G-2)	DQ418784	90	DQ418784	87	DQ418787	89	4a
Pak-24-10 (G-2)	DQ418783	99	DQ988073	89	DQ418784	80	4a

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Pak-25-10 (G-2)	DQ418784	92	DQ516084	90	DQ418782	84	4a
Pak-26-10 (G-2)	DQ418788	97	DQ418789	91	DQ418783	92	4a
Pak-27-10 (G-2)	Y11604	96	DQ988079	93	DQ516084	98	4a
Pak-28-10 (G-2)	AY766510	96	FJ462436	93	EF116123	99	4c
Pak-29-10 (G-2)	EF115681	98	FJ462436	94	GU088166	90	4c
Pak-30-10 (G-2)	AY766558	93	GU088132	98	GU088167	91	4c
Pak-31-10 (G-2)	AF064490	92	DQ418789	89	GU088166	93	5a
Pak-32-10 (G-2)	JQ318085	91	Y13184	88	NC_009826	92	5a
Pak-33-10 (G-2)	JQ318156	92	HM566118.	90	HM566115	93	5a
Pak-34-10 (G-2)	JQ318156	90	HM566116	91	HM566117	94	5a
Pak-35-10 (G-2)	EF424625	97	EF424625	94	EF424625	97	6q
Pak-36-10 (G-2)	EU246931	96	EU246932	89	EU408326	96	6e
Pak-37-10 (G-2)	FJ025854	95	FJ025855	89	JQ318231	98	4b
Pak-38-06 (G-2)	FJ025856	90	JQ318289	90	FJ025855	91	4b
Pak-39-10 (G-2)	JQ318044	89	FJ462435	91	FJ025856	3	4b
Pak-40-10 (G-2)	JQ318102	98	FJ025854	92	FJ025855	4	4b
Pak-41-10 (G-2)	JQ318156	88	DQ418789	92	GU088166	93	Unresolved
Pak-42-06 (G-2)	AB047639	87	AB047645	93	AF169004	93	2a
Pak-43-10 (G-2)	AB047643	98	AF238481	99	AF169005	98	2a
Pak-44-10 (G-2)	D50409	97	JX227965	89	JX227951	89	2c
Pak-45-10 (G-2)	JX227949.	91	JX227950	98	JX227966	87	2c
Pak-46-10 (G-2)	AB661375	95	AB661373	87	AB661380	88	2b
Pak-47-10 (G-2)	AB661379	94	AB661382	87	AB661376	89	2b
Pak-48-10 (G-2)	AB661390	90	AB661391	86	AB661395	90	2b
Pak-49-10 (G-2)	AB661400	91	AB661402	98	AB661400	91	2b
Pak-50-10 (G-2)	AB661399	93	AB661401	90	AB661389	92	2b
Pak-51-10 (G-2)	AB049087	95	AB049089	91	AB049088	90	1b

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Pak-52-10 (G-2)	EU158186	96	EU798761	93	FJ435090	92	6v
Pak-53-10 (G-2)	AY051292	89	D14853	92	AY651061	99	1c
Pak-54-10 (G-2)	AY651061	87	GU441320	94	D14853	93	1c
Pak-55-10 (G-2)	D14853	89	GU441322	98	HQ833247	94	1c
Pak-56-10 (G-2)	GU441334	87	GU441326	97	HQ833227	95	1c
Pak-57-10 (G-2)	AB691595	89	AY070186	90	AY070194	90	3c
Pak-58-10 (G-2)	AB691596	89	NC_009824.	97	JQ318205	93	3c
Pak-59-10 (G-2)	JQ318100	90	AY070194	90	AY070192	90	3c
Pak-60-10 (G-2)	JX944790	98	KC127626	91	GQ867012	98	5b
Pak-61-10 (G-2)	AB661399	91	JX944790	90	GQ867006	9	5b
Pak-62-10 (G-2)	GU441334	97	AB049089	93	GQ867010	93	5b
Pak-63-10 (G-2)	JX227949	90	AB049089	92	GQ867008.	93	5b
Pak-64-10 (G-2)	JQ341409	89	DQ480523.	93	JX227966	98	6a
Pak-65-10 (G-2)	DQ480522	88	JQ318124	94	DQ480521	97	6a
Pak-66-10 (G-2)	DQ480521	89	JQ318041	95	DQ480513	90	6a
Pak-67-10 (G-2)	DQ480518.	87	DQ480517.	93	DQ480512	92	6a
Pak-68-10 (G-2)	JQ318124.	86	DQ480514	90	D14853	93	6a
Pak-69-10 (G-2)	EU686683	85	AB691595	91	AY070186	92	3a
Pak-70-10 (G-2)	AB691596	92	AY070188	90	AY070180	94	3a
Pak-71-10 (G-2)	JQ318205	91	AY070187	98	AY070192	92	3a
Pak-72-10 (G-2)	JQ318199	93	AY070183	87	AF046866	91	3a
Pak-73-10 (G-2)	AB470111	93	AB470142	86	AB4700	90	Unresolved
Pak-74-10 (G-2)	AB470112	90	AB470142	85	AB470010	93	Unresolved
Pak-75-10 (G-2)	AB470113	98	AB470143	97	AB470018	92	Unresolved

3.2.5.7 Untypeable genotype

In group 1 (SVR) genotypes of all the samples were resolved while among patients of group 2 (non- responders) 5 sample remain unresolved.

3.2.6 Relationship of Genotypes with Demographics of Patients

The route of HCV transmission was evaluated in relation with the genotypes in patients of both the groups 1 and 2. The patients were divided into two subgroups according to their age. All patients in group 1 were below 42 years. Among the selected patients of group 1 (with SVR) genotype 3a was predominant and two main routes of HCV transmission were found, includes blood or blood products and sexual transmission. Among the patients undergone the invasive procedures, 2 had a history of surgery, 2 of piercing or tattoos, 2 of acupuncture, 3 had dental therapy and 1 had endoscopy and genotype 3a is predominant. It was observed that patients with intravenous drug abuse were infected mainly with genotype 3a and 3b (Table 3.2.3).

Majority of the patients in group 2 were more than 42 years in age. Moreover the main source of HCV infection was similar to group 1 i.e blood or blood products followed by IDUs in non-responders (group 2) with spread of diverse range of genotypes. Among the patients with invasive procedures 3 had surgery, 1 had piercing injury, 3 had acupuncture and 10 had dental therapy with prevalence of genotypes 2, 3 4 and 6. Patients who were injection drug users were 19 in number, exhibited genotype 1, 2, 3 4 and 5 while patients with unresolved genotypes includes 2 patients infected with acupuncture, 1 was sexually transmitted, 1 with blood or blood products and 1 with unknown source (Table 3.2.4).

3.3 EVOLUTIONARY DYNAMICS AND HCV RESPONSE TO ANTIVIRAL THERAPY

3.3.1 Determination of Phylogeny

In the current study in order to determine the role of genetic diversity of HCV isolates in antiviral response, phylogenetic trees were constructed on the sequences of 5′ UTR, core and NS5B separately in association with the sequences from all over the world for both the treatment groups using the maximum parsimony (MP) algorithm of MEGA 5

Table 3.2.3: Demographic Characteristics of patient's in group 1 in relation to their genotypes.

Demographics of Patients	Genotypes n (%)				P Values
	1b	3a	3b	4b	
Patients (N)	10	20	36	9	
Male/Female	5/5	15/5	20/16	6/3	<0.036
Age Years	35 ± 7.65	41 ± 5.43	40 ± 6.32	37 ± 4.32	<0.040
Mode of Transmission					
Unknown (N=9)	1 (11.1)	3 (33)	4 (44.4)	1 (11.1)	<0.066
Blood or Blood Product (N=20)	4 (20)	3 (15)	11 (55)	2 (10)	<0.000
IDU (N=16)	2 (12.5)	4 (25)	8 (50)	2 (12.5)	<0.067
Invasive Procedure					
Surgery (N=2)	1 (50)	1 (50)	0 (0.0)	0 (0.0)	<0.02
Piercing Injury (N=2)	0 (0.0)	0 (0.0)	2 (100)	0 (0.0)	<0.001
Acupuncture (N=2)	0 (0.0)	1 (50)	1(50)	0 (0.0)	<0.064
Needle Injury (N=4)	0 (0.0)	2 (50)	1(25)	1 (25)	<0.058
Dental Therapy (N=9)	1 (11.1)	3 (33)	4(44)	1 (11)	<0.001
Endoscopy (N=1)	0 (0.0)	1(100)	0 (0.0)	0 (0.0)	<0.001
Sexual Transmission (N=10)	1 (10)	2 (20)	5 (50)	2 (20)	<0.001

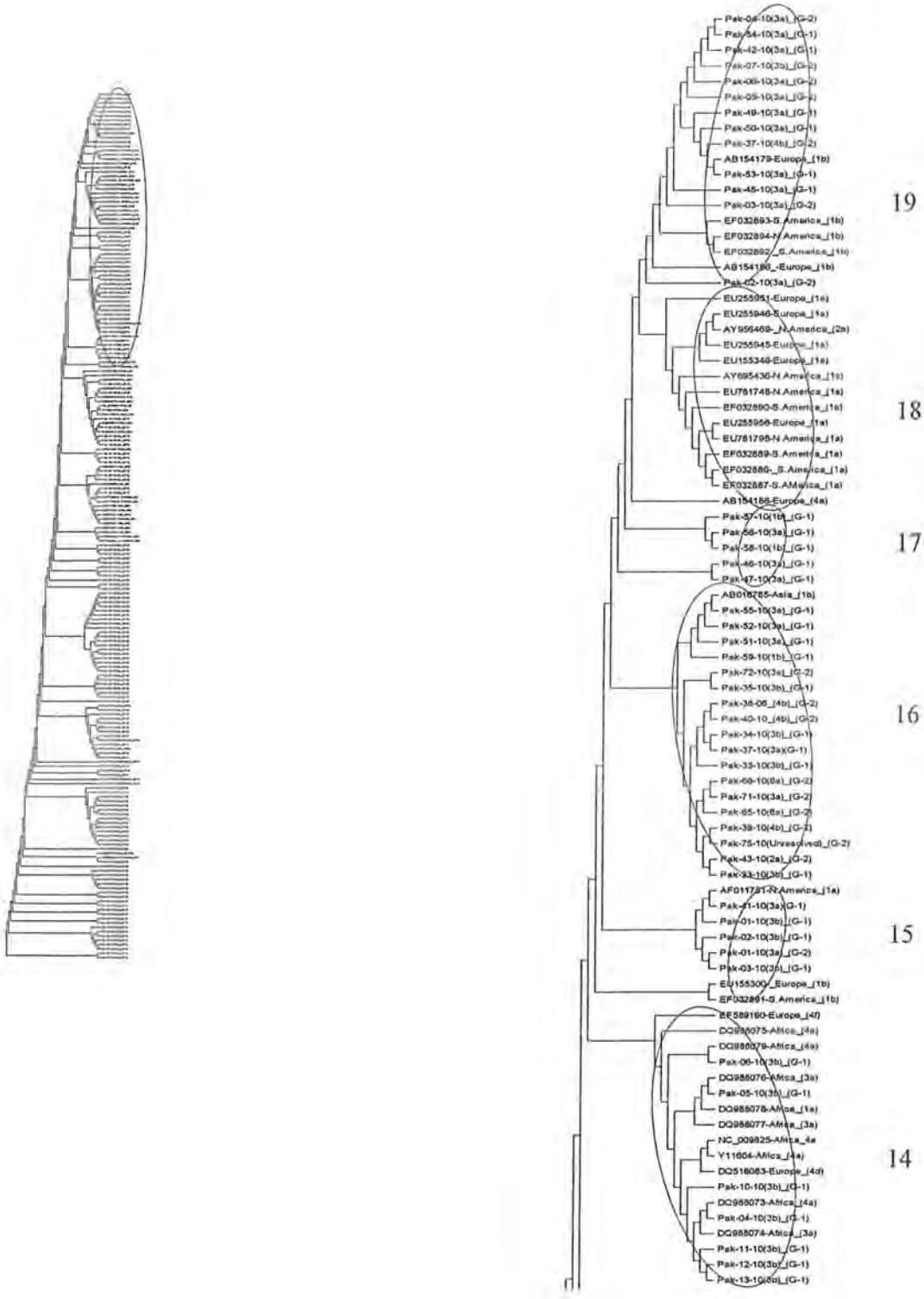
Table 3.2.4: Demographic Characteristics of patient's in group 2 in relation to their genotypes

Clinical Parameters	Genotypes n (%)																		P Value
	1a	1b	1c	2a	2b	2c	3a	3b	3c	4a	4b	4c	5a	5b	6a	6q	6e	6v	Untyp-eable	
Patients (N)	5	8	4	2	5	2	10	2	3	6	4	3	4	4	5	1	1	1	5	
Male/ Female	2/3	5/3	3/1	1/1	3/2	0/2	7/3	1/2	2/1	3/3	2/2	1/2	3/1	1/3	3/2	0/1	1/0	1/0	3/2	< 0.002
Age Years	25.3 ±4.4	50.2 ±10	40.2 ± 8.4	55.0 ±9.3	40.4 ±8.7	45.8 ±6.6	47.5 ±8.7	38.3 ±6.6	47.2 ±7.7	55.8 ±5.4	58.1 ±8.9	48.8 ±9.6	40.0 ±8.7	57.4± 7.8	59.3 ±11.4	60.1 ±10.1	56.4 ± 12.3	55.5 ±10.2	50.4 ±9.3	< 0.001
Mode of Transmission																				
Unknown (N= 5)	1 (20)	1 (20)	2 (40)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (20)	< 0.02
Blood or Blood Product (N= 24)	2 (8.3)	3 (12.5)	2 (8.3)	1 (4.1)	0 (0.0)	0 (0.0)	1 (4.1)	1 (4.1)	1 (4.1)	2 (8.3)	2 (8.3)	1 (4.1)	2 (8.3)	2 (8.3)	2 (8.3)	0 (0.0)	1 (4.1)	0 (0.0)	1 (4.1)	< 0.039
IDU (N= 19)	1 (5.2)	3 (15.7)	0 (0.0)	1 (0.0)	1 (5.2)	1 (5.2)	6 (31.5)	1 (5.2)	1 (5.2)	1 (5.2)	1 (5.2)	0 (0.0)	0 (0.0)	1 (5.2)	1 (5.2)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	< 0.042
Invasive Procedure																				
Surgery (N= 3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (33)	0 (0.0)	0 (0.0)	2 (66.6)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	< 0.002
Piercing Injury (N= 1)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (100)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	< 0.002
Acupuncture (N= 3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (33)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (66.6)	< 0.003
Needle Injury (N= 3)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (33)	0 (0.0)	1 (33)	0 (0.0)	0 (0.0)	1 (33)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	< 0.002
Dental Therapy (N= 10)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (10)	1 (10)	1 (10)	0 (0.0)	0 (0.0)	2 (20)	1 (10)	1 (10)	0 (0.0)	1 (10)	0 (0.0)	1 (10)	0 (0.0)	1 (10)	0 (0.0)	< 0.003
Endoscopy (N= 0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0	0 (0.0)	-
Sexual Transmission (N= 7)	1 (14)	1 (14)	0 (0.0)	0 (0.0)	1 (14)	0 (0.0)	1 (14)	0 (0.0)	1 (14)	0 (0.0)	0 (0.0)	0 (0.0)	1 (14)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (14)	< 0.003

The sequences of all the three genomic regions of both the treatment groups were successfully aligned by using online multiple alignment software ClustalW. Aligned sequences were further subjected to construct tree using the software MEGA 5. Evolutionary trees of sequenced samples of both treatment groups i.e those who respond and those who remain non-responders, were constructed to find out the relationships among the sequences, novel genotypes, subtypes and variants. Closely related HCV sequences reported from five continents around the globe were retrieved from Los Alamos National Laboratory (LANL) HCV database to find out the evolutionary relationship between the studied strains and the HCV strains circulating in these continents.

Phylogenetic tree constructed by maximum parsimony method using sequences of 5′ UTR from both treatment groups exhibiting the mixed pattern of evolution by both the treatment groups (Figure 3.3.1). Overall the tree was divided into 16 clusters. Cluster 1 and 2 constitute some sequences of group 1 depicting the early evolution of the sequences from patients who responded positively to therapy. Moreover sequences from group 1 are present in almost all the clusters of the tree. Majority of the sequences from the group 2 are present in the recently evolved clusters (clusters 12, 15, 16 and 19). Most of the sequences from group 2 make their separate clusters while somewhere in the tree they possess the position along with the reference sequences from the database (clusters 6 and 12). The unresolved genotypes are found in close neighbor to genotype 2, 3, 4 and 6 (clusters 5, 6 and 16).

Evolutionary tree constructed using MP method on the basis of core sequences exhibited the similar branching pattern of group 1 and 2. Overall the tree was divided into 15 clusters (Figure 3.2.2). Some sequences from group 1 and 2 are found at the base of the tree and exhibited the early evolutionary period (cluster 1). In cluster 2 sequences from group 1 showed close homology with Asian isolates. At some positions throughout the tree topology some of the sequences from both groups are clustered with each other (clusters 4 and 10) while in others clusters they were found in close proximity with reference strains throughout the world (clusters 2, 3, 6, 7, 8, 9, 11, and 14).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Pak-54-10(3a)_(G-1)
Pak-42-10(3a)_(G-1)
Pak-07-10(3b)_(G-2)
Pak-06-10(3a)_(G-2)
Pak-05-10(3a)_(G-2)
Pak-49-10(3a)_(G-1)
Pak-50-10(3a)_(G-1)
Pak-37-10(4b)_(G-2)
AB154179-Europe_(1b)
Pak-53-10(3a)_(G-1)
Pak-45-10(3a)_(G-1)
Pak-03-10(3a)_(G-2)
EF032893-S.America_(1b)
EF032894-N.America_(1b)
EF032892_S.America_(1b)
AB154180_-Europe_(1b)
Pak-02-10(3a)_(G-2)
EU255951-Europe_(1a)
EU255946-Europe_(1a)
AY956469-_N.America_(2b)
EU255945-Europe_(1a)
EU155348-Europe_(1a)
AY695436-N.America_(1a)
EU781748-N.America_(1a)
EF032890-S.America_(1a)
EU255956-Europe_(1a)
EU781795-N.America_(1a)
EF032889-S.America_(1a)
EF032888-_S.America_(1a)
EF032887-S.AMerica_(1a)
AB154186-Europe_(4a)
Pak-57-10(1b)_(G-1)
Pak-56-10(3a)_(G-1)
Pak-58-10(1b)_(G-1)
Pak-46-10(3a)_(G-1)
Pak-47-10(3a)_(G-1)
AB016785-Asia_(1b)
Pak-55-10(3a)_(G-1)
Pak-52-10(3a)_(G-1)
Pak-51-10(3a)_(G-1)
Pak-59-10(1b)_(G-1)
Pak-72-10(3a)_(G-2)
Pak-35-10(3b)_(G-1)
Pak-36-06_(4b)_(G-2)
Pak-40-10_(4b)_(G-2)
Pak-34-10(3b)_(G-1)
Pak-37-10(3a)(G-1)
Pak-33-10(3b)_(G-1)
Pak-68-10(6a)_(G-2)
Pak-71-10(3a)_(G-2)
Pak-65-10(6a)_(G-2)
Pak-39-10(4b)_(G-2)
Pak-75-10(Unresolved)_(G-2)
Pak-43-10(2a)_(G-2)
Pak-31-10(3b)_(G-1)
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Pak-01-10(3b)_(G-1)
Pak-02-10(3b)_(G-1)
Pak-01-10(3a)_(G-2)
Pak-03-10(3b)_(G-1)
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EF032891-S.America_(1b)
EF589160-Europe_(4f)
DQ988075-Africa_(4a)
DQ988079-Africa_(4a)
Pak-06-10(3b)_(G-1)
DQ988076-Africa_(3a)
Pak-05-10(3b)_(G-1)
DQ988078-Africa_(1a)
DQ988077-Africa_(3a)
NC_009825-Africa_4a
Y11604-Africa_(4a)
DQ516083-Europe_(4d)
Pak-10-10(3b)_(G-1)
DQ988073-Africa_(4a)
Pak-04-10(3b)_(G-1)
DQ988074-Africa_(3a)
Pak-11-10(3b)_(G-1)
Pak-12-10(3b)_(G-1)
Pak-13-10(3b)_(G-1)
19
18
17
16
15
14

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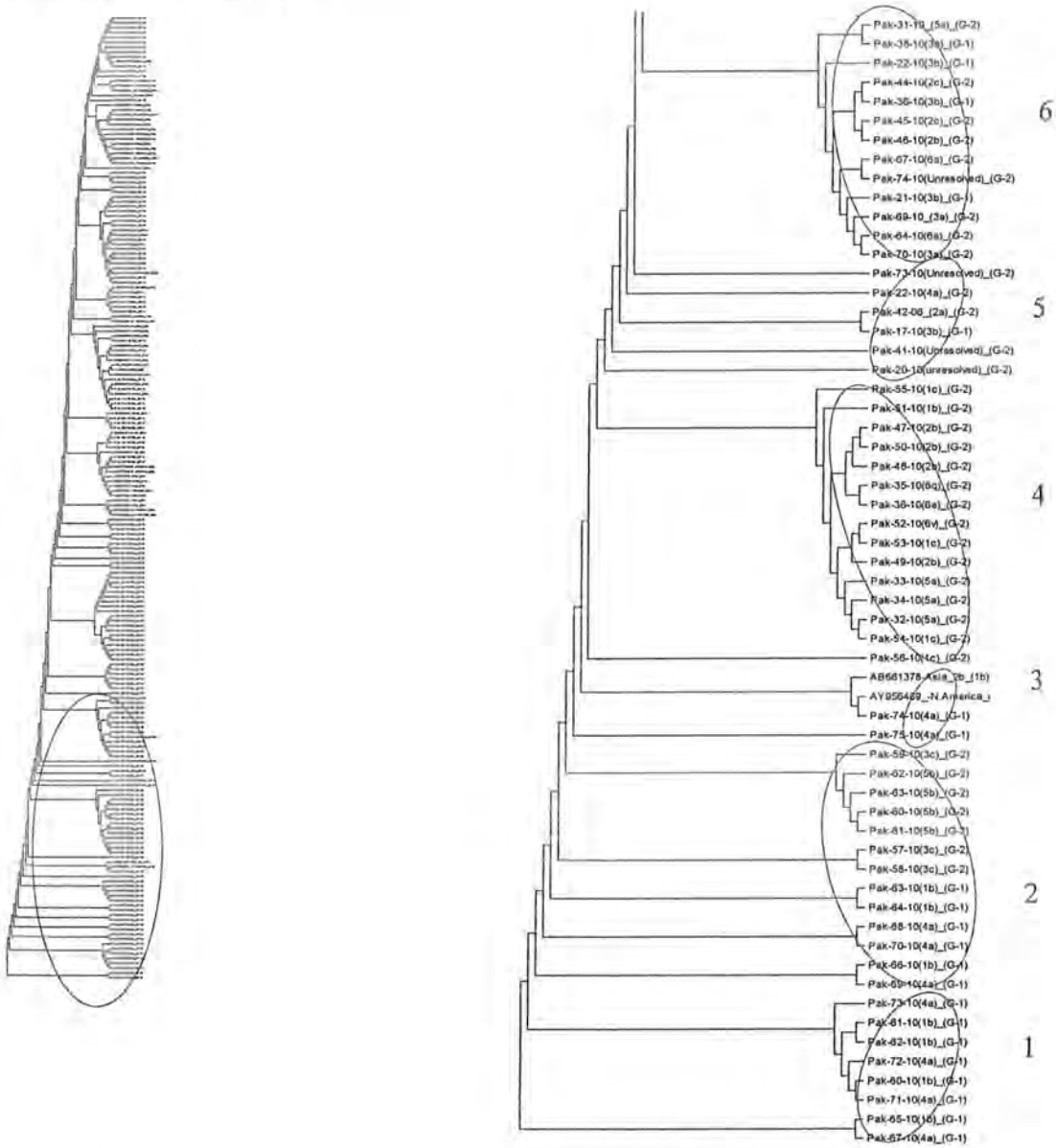
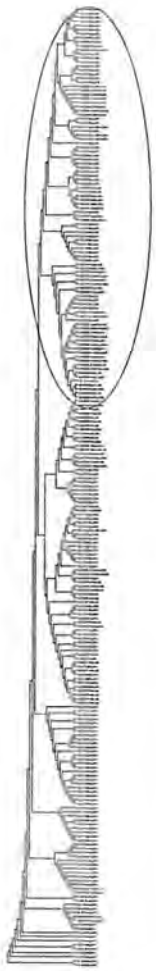
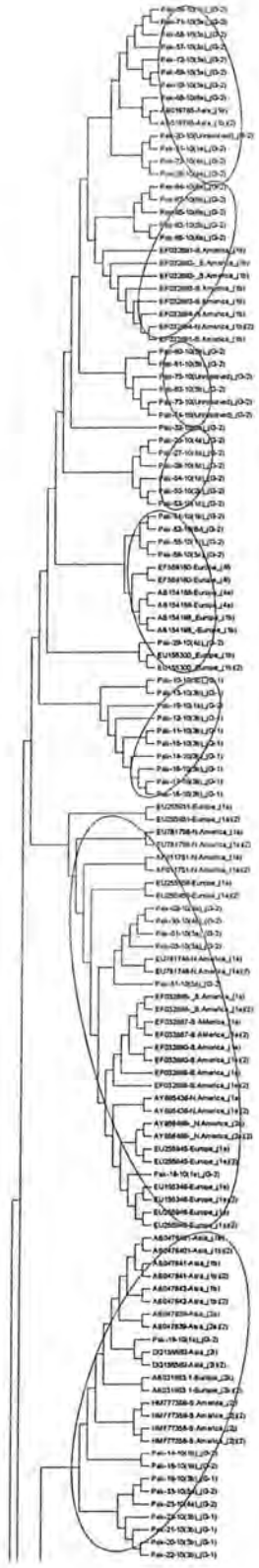


Figure 3.3.1: Parsimonious phylogenetic tree constructed using 5'UTR sequences of patients from both groups with reference sequences from database.





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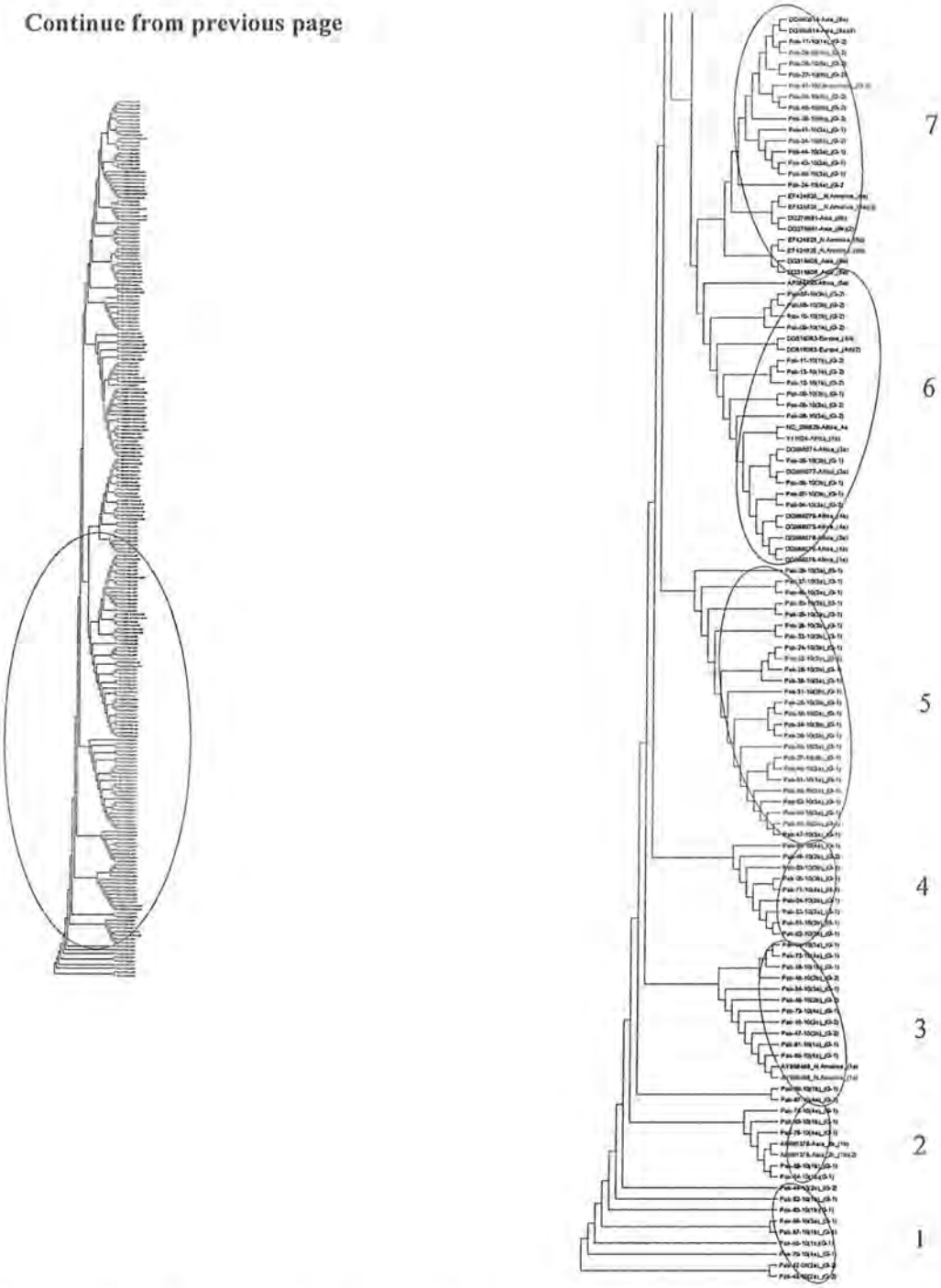

Figure 3.3.2: Parsimonious phylogenetic tree constructed using core sequences of patients from both groups with reference sequences from database.

Three out of 5 unresolved genotypes are present in cluster 13 and in neighbor of genotype 5 while one unresolved strain was found in cluster 15 along with the genotype 1 and another unresolved strain took the place in cluster 7 along with genotype 4 (Figure 3.3.2).

Using MP method dendrogram constructed with sequences from NS5B exhibited diverge branching pattern by almost all the sequences from both groups 1 and 2. Tree was divided into 10 clusters (Figure 3.3.3). Sequences from group 1 and 2 along with the reference sequences from Europe are positioned at the base of the tree exhibiting the early evolutionary behavior. Most of the sequences from group 2 were depicting the recent evolution (cluster 9 and 10). Some sequences from group 1 and 2 presenting the homology with sequences from Africa, Asia, America (North and South) and Europe (cluster 2, 3, 5 and 7). Moreover the unresolved sequences showed homology with genotypes 1, 3, 4, 5 and 6 (cluster 6, 8, 9 and 10) (Figure 3.3.3). Some strains did not show any branching pattern giving an idea about the absence of any change that has occurred in them for years. This might be due to the high negative selection pressure which they are undergoing due to some environmental factors whereas their non-response to the therapy might be due to some other change at nucleotide or amino acid level or some host factors involved. Therefore, here it can be proposed that less diversification leads to positive response towards antiviral therapy.

3.3.2 Nucleotide Sequence Diversity of 5′ UTR, CORE AND NS5B

Sequence diversity of all the three concerned genomic regions (5′ UTR, core and NS5B) of the present study was found using BioEdit version 7.3 for both the treatment groups. It was observed that patients who responded treatment (SVR) had significantly less number of transitions and genetic distances than the non-responders (Table 3.3.1). It is also shown that the total number of mutation spots (including all the insertions, deletions, transitions and transversions) were less among responders (group 1) than in the non-responders (group 2). The targeted genomic regions (5′ UTR, core and NS5B) in the present study also showed more non-synonymous substitutions than synonymous in group 2 (non-responders) as compared to group 1 (with SVR).

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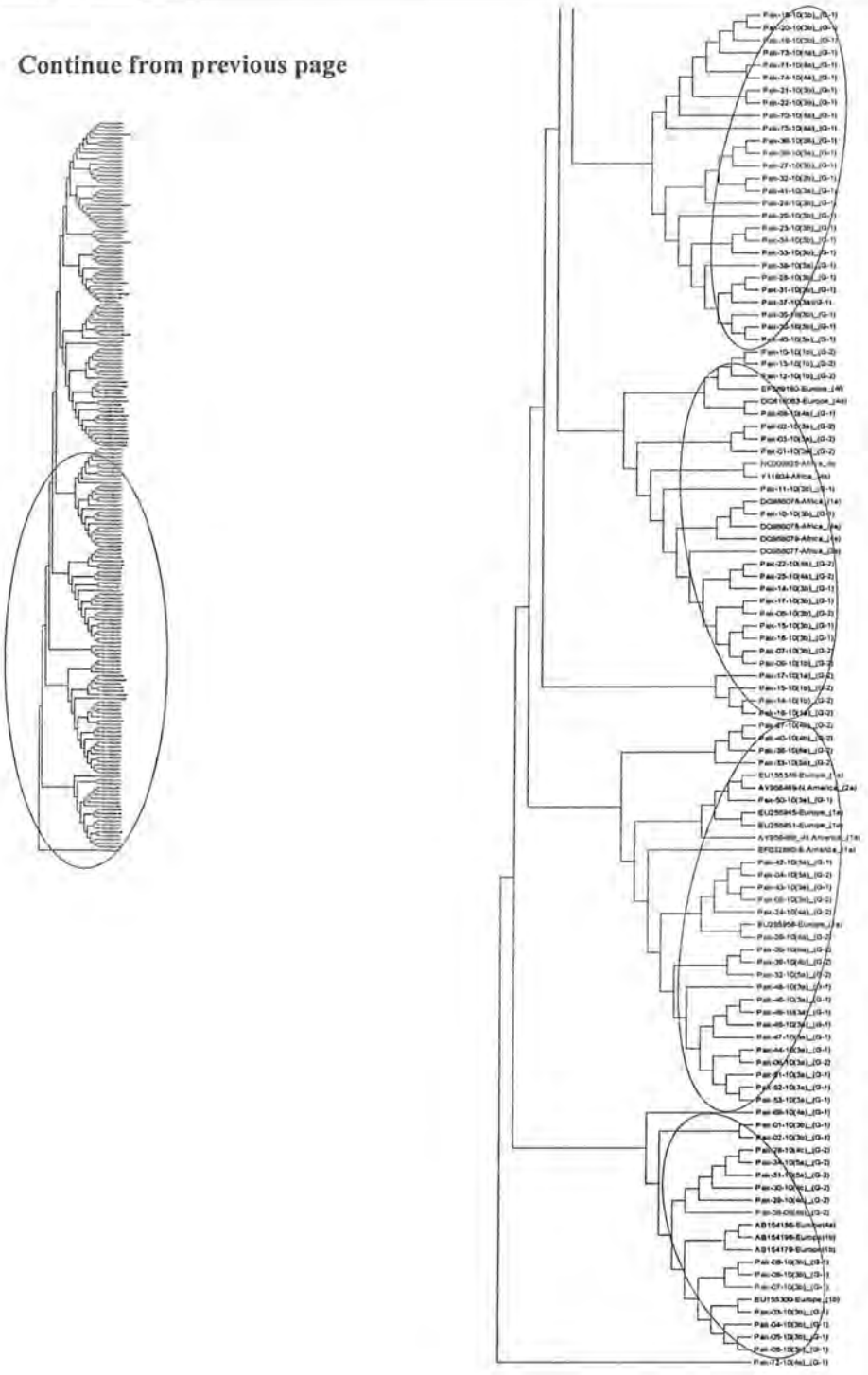


Figure 3.3.3: Parsimonious phylogenetic tree constructed using NS5B sequences of patients from both groups with reference sequences from database.

Table 3.3.1: Genetic diversity of all the three genomic regions (5′ UTR, core and NS5B) of HCV isolates for both treatment groups.

Factors	5′ UTR			Core			NS5B		
	Group 1 (n=75)	Group 2 (n=75)	P Values	Group 1 (n=75)	Group 2 (n=75)	P Values	Group 1 (n=75)	Group 2 (n=75)	P Values
Insertions	0.33 ± 0.52	1.08 ± 1.9	< 0.001	0.23 ± 0.521	0.05 ± 1.1	< 0.001	0.34 ± 0.52	1.18 ± 1.9	< 0.001
Deletions	0.66 ± 0.82	1.2 ± 0.59	< 0.002	0.65 ± 0.22	1.1 ± 0.53	< 0.001	0.61 ± 0.20	1.0 ± 0.10	< 0.030
Transitions	1.8 ± 0.9	4.7 ± 2.0	< 0.001	1.7 ± 0.4	4.1 ± 1.0	< 0.003	2.0 ± 0.7	4.8 ± 1.6	< 0.015
Transversions	2.0 ± 2.5	2.5 ± 2.1	< 0.051	1.9 ± 0.5	2.4 ± 0.1	< 0.001	1.8 ± 0.3	1.8 ± 1.1	< 0.073
No. of Mutation Spots	6.2 ± 3.1	8.2 ± 4.2	< 0.043	3.1 ± 0.3	7.2 ± 4.0	< 0.002	9.2 ± 2.1	11.2 ± 4.2	< 0.004
Genetic Distance	0.005 ± 0.24	0.13 ± 0.75	< 0.001	0.007 ± 0.31	0.11 ± 0.21	< 0.003	0.001 ± 0.25	0.17 ± 0.27	< 0.001
Non-Synonymous Substitution	0.07 ± 0.21	0.09 ± 0.13	< 0.050	0.007 ± 0.10	0.07 ± 0.03	< 0.001	0.15 ± 0.11	0.35 ± 0.14	< 0.031
Synonymous Substitutions	0.005 ± 0.20	0.006 ± 0.01	< 0.053	0.001 ± 0.02	0.01 ± 0.01	< 0.031	0.004 ± 0.12	0.006 ± 0.12	< 0.030

Group 1: Patients with SVR
Group 2: Non-responders

The higher rates of non-synonymous mutations are considered to modify the epitopes and help the virus to evade both the treatment and immune system. Present results showed the occurrence of more variability in sequences of NS5B as compared to the sequences of 5′ UTR and core. Our findings showed that nucleotides 30–170 of 5′ UTR are highly conserved between the two studied groups as compared to the other two genomic regions (Table 3.3.2).

3.3.3 3D Structural and PROCHECK Analysis

Protein models for the studied sequences were first built using i-TASSER i.e an online platform that predict protein structure and function and generates up to five full-length atomic models (ranked based on cluster density). Figure 3.3.4 and 3.3.5 demonstrating the representative models of core and NS5B obtained from i-TASSER picked randomly among the 75 sequences each for both treatment groups and for both proteins.

3D models were further evaluated on PROCHECK. Obtained output in the form of Ramachandran plots for core and NS5B protein models showed whether the amino acid residues exist in the "favored region" or "disallowed region" of the plot (Figure 3.3.6 and 3.3.7). For a good protein model there must be ≥ 80 residues in the most favored region or < 2 in the disallowed region of the plot. Models predicted by i-TASSER with highest C-score as well as satisfactory plot statistics (< 75) can be further used for 3D models analysis by using different tools like Rasmol. 3D structural models of HCV can help us predicting the cleavage sites or recognition of phoshorylation sites.

3.3.4 Frequency of Amino Acids

The amino acid sequences of core and NS5B were aligned and frequency of amino acid was determined using MEGA 5. Percentage of the individual amino acids in both core and NS5B were compared between the two groups (1 and 2). Some of the amino acids were equally frequent among all the samples while some showed the considerable divergence among the two groups. Amino acids, alanine, glycine, proline, arginine and serine showed remarkable difference between the core sequences of the both groups (Figure 3.3.8A).

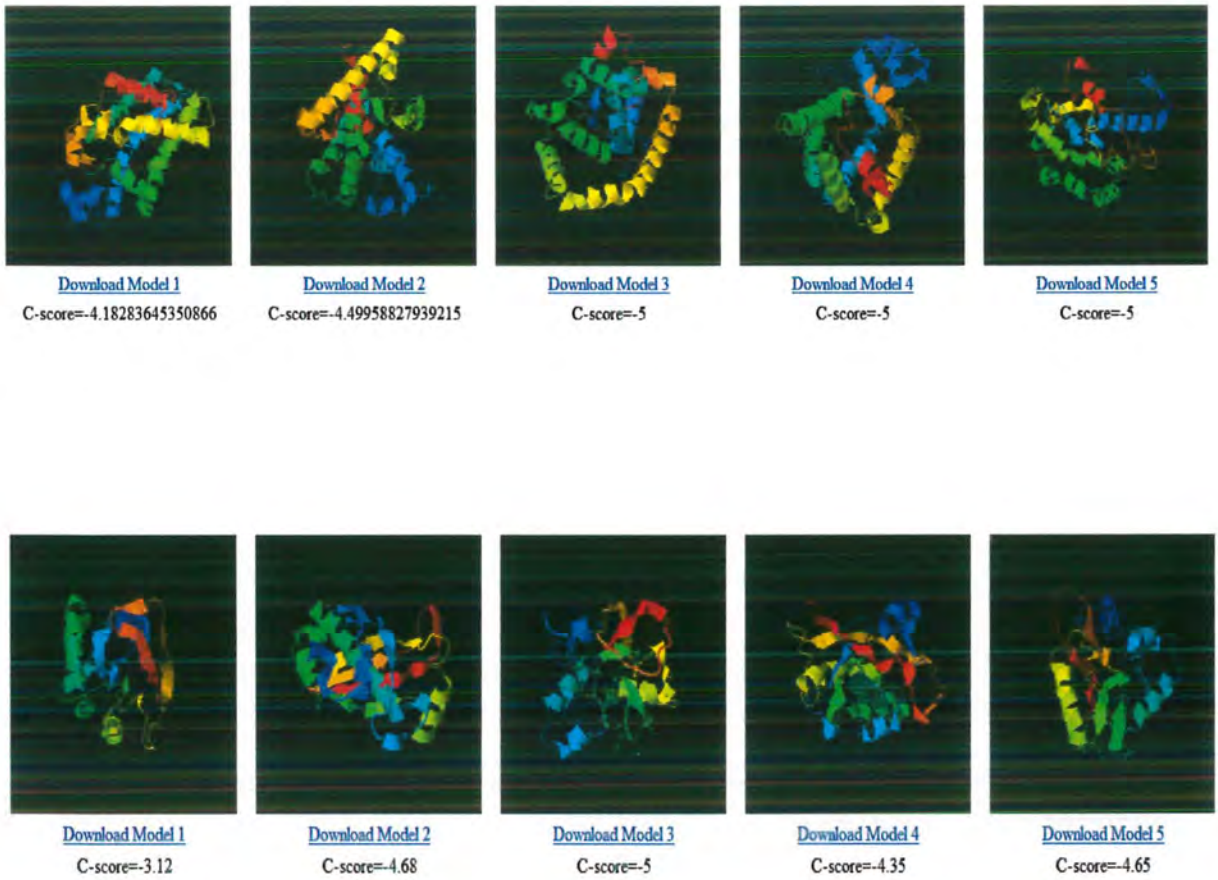


Figure 3.3.4: Representative top models built by i-TASSER for the sequences of core protein for (A) group 1 (with SVR)and (B) group 2 (non-responders). C-score from i-TASSER that reveals the strength of predicted models.

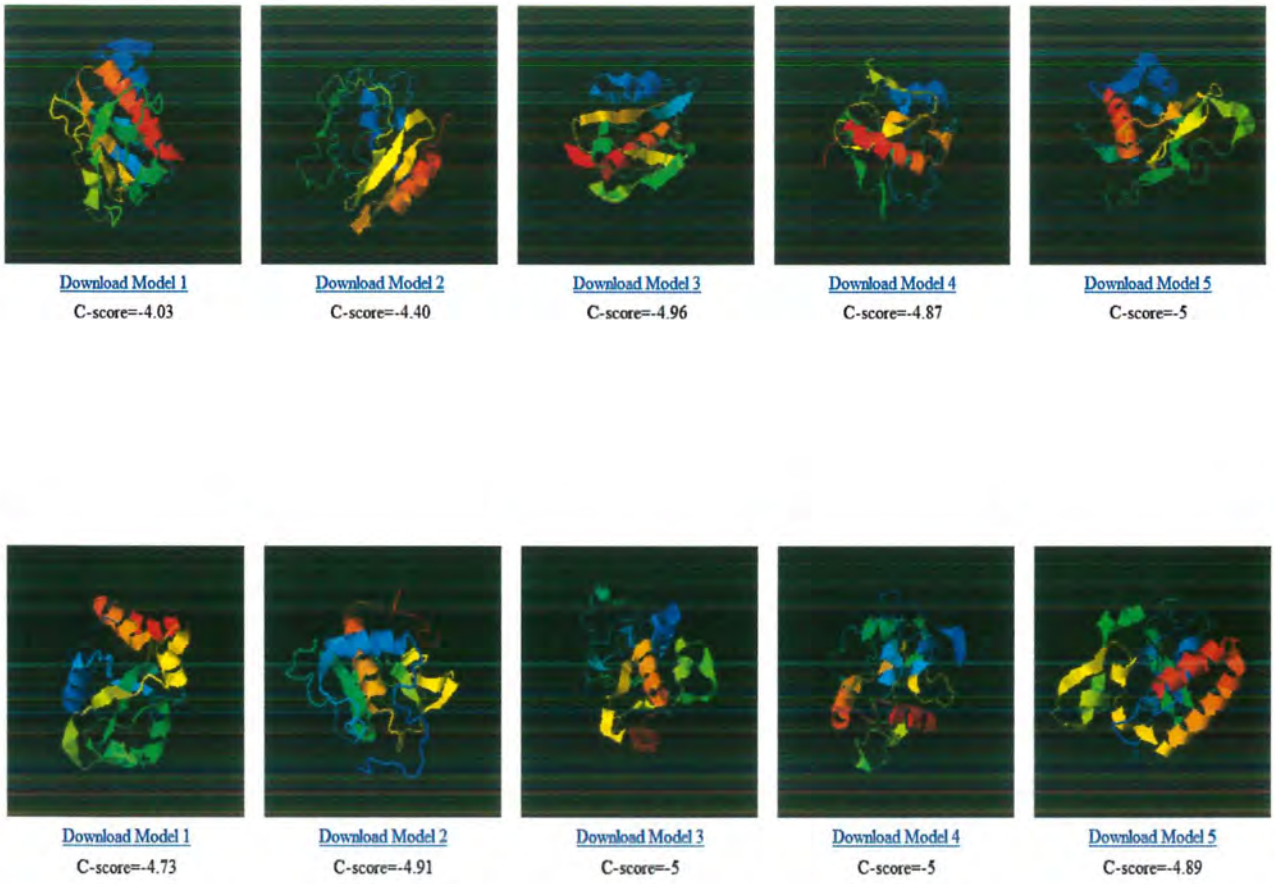


Figure 3.3.5: Representative top models built by i-TASSER for the sequences of NS5B protein for (A) group 1 (with SVR) and (B) group 2 (non-responders). C-score from i-TASSER that reveals the strength of the predicted models.

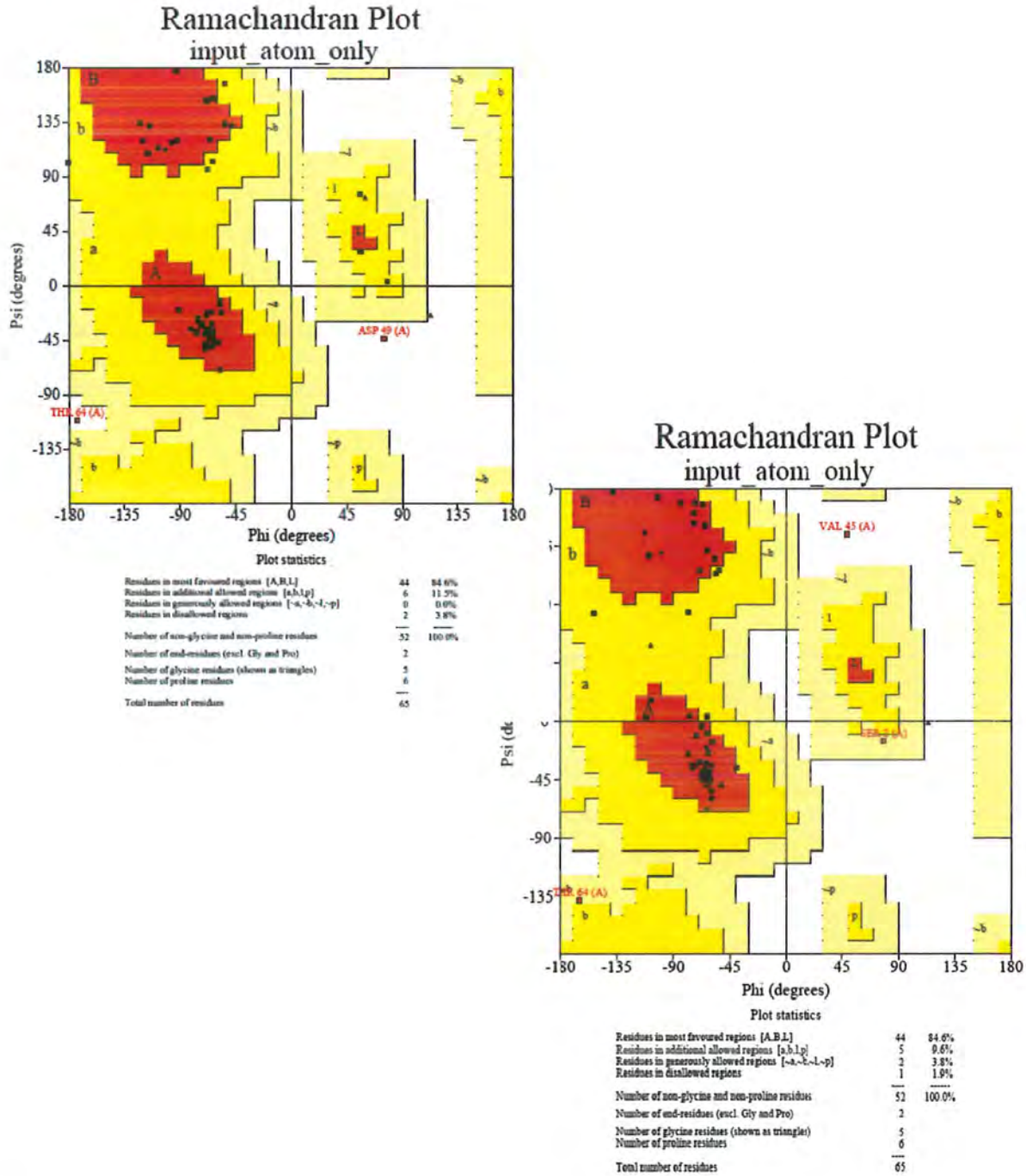


Figure 3.3.6: Representative Ramchandran plots for core protein from (A) group 1 (with SVR) and (B) group 2 (non-responders).

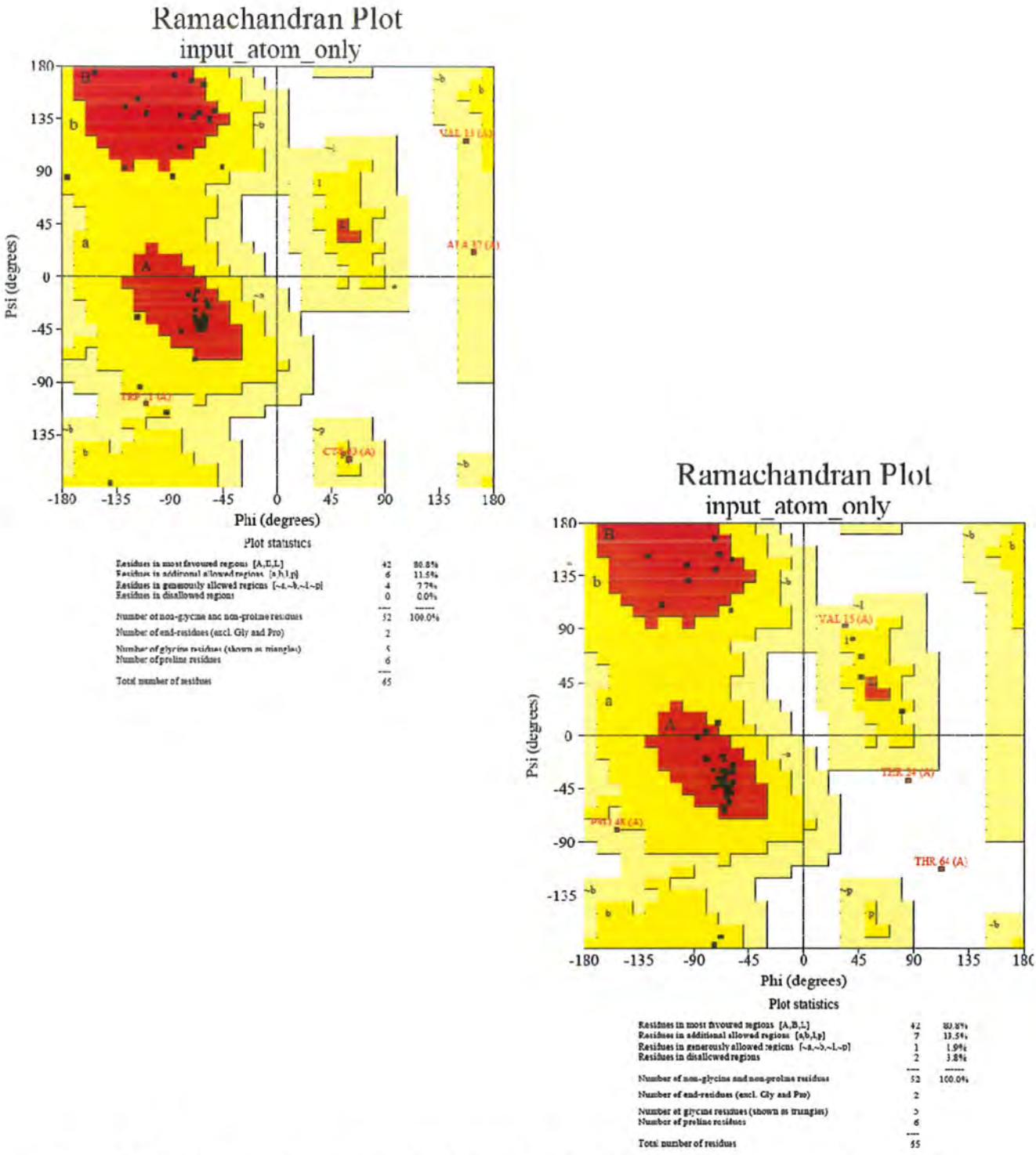


Figure 3.3.7: Representative Ramchandran plots for NS5B protein from (A) group 1 (with SVR)and (B) group 2 (non-responders).

When the amino acid sequences of NS5B were compared between both groups that showed a considerable change in amino acids i.e., alanine, aspirgilin, glycine, histidine, isoleucine, proline, arginine and threonine (Figure 3.3.8B).

3.3.5 Analysis of Amino Acid Heterogeneity

The output of the MEGA 5 and DNAman revealed that total number of conserved and variable sites was considerably different in both treatment groups with the difference in amino acid positions. The conserved amino acid positions in SVR patients showed substitution in amino acids in non-responders (Table 3.3.3). Multiple amino acid substitution at single site was more common in non-responders than in SVR patients for example at position 1 and 4 amino acids histidine and threonine were replaced by four other amino acids in non-responders whereas in SVR patients at these positions single substitutions were observed. Graphical output of some of the amino acid substitution is given in the figure 3.3.9.

3.3.6 Prediction of N-Linked Phosphorylation Site

In silico N-linked phosphorylation sites were predicted using NetPhos server 2.0. It was observed that amino acid sequences of core protein in non-responders (group 2) exhibit more potential phosphorylation sites as compared to those shown by sequences of patients with SVR (group 1) with p value of < 0.001. Moreover the amino acid sequences of NS5B did not show any considerable difference in number of predicted phosphorylation sites between the two groups ($P < 0.070$). Representative predictions among the 75 sequences each for both groups and also for both proteins are given in figure 3.3.10 and 3.3.11. Predictions were made on the "Low Stringency" to identify as many sites as possible.

3.3.7 Determination of N-Linked Glycosylation Sites

An increase in N-linked glycosylation sites was observed in core ($P < 0.003$) and NS5B ($P < 0.002$) protein sequences in patients who did not respond to the therapy (group 2) as compared to those who showed SVR (group 1) (Figure 3.3.12 and 3.3.13).

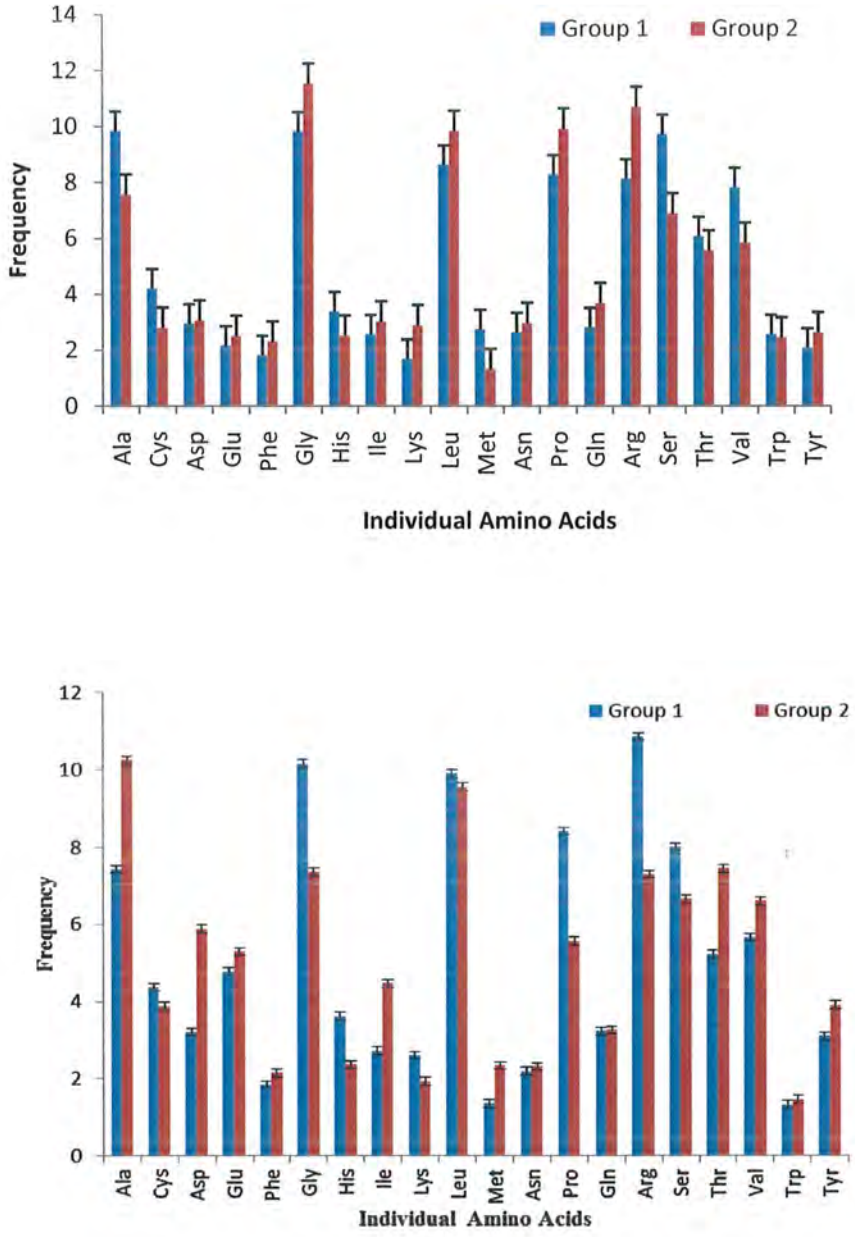


Figure 3.3.8: Comparison of amino acid frequency between the two groups; (A) Core and (B) NS5B.

Table 3.3.3: Conserved and variable regions of HCV in the amino acid sequences of core and NS5B among the two groups.

Treatment Groups	Conserved Amino Acids Positions		P Value	Variable Amino Acids Positions		P Value
	Core	NS5B		Core	NS5B	
Group 1 (n=75)	3, 9, 10, 15,21,23, 25, 28, 35, 105, 106, 123, 134, 136, 137, 142	14,18,65,12 0,133,141	< 0.001	24,29,34,36, 38,39, 41, 65, 72, 85, 112,141, 151	35,43,44,47,48,5 1,59,62,65, 68, 70, 87,89,95, 102, 107, 112, 118, 120, 129, 221, 240, 256	0.002
Group 2 (n=75)	3, 7, 9, 25, 28, 35, 106, 165, 205	13, 18, 24, 67, 85, 109, 120, 134, 143, 154, 153	< 0.001	23, 29, 35, 37, 38, 41, 43, 54, 56, 67, 72, 78, 85, 105, 112, 120, 135, 141, 156	26, 35, 48, 95, 105, 109, 108, 114, 118, 132, 143, 154, 165, 163, 172, 187, 201, 221, 240, 289, 267,256	< 0.03

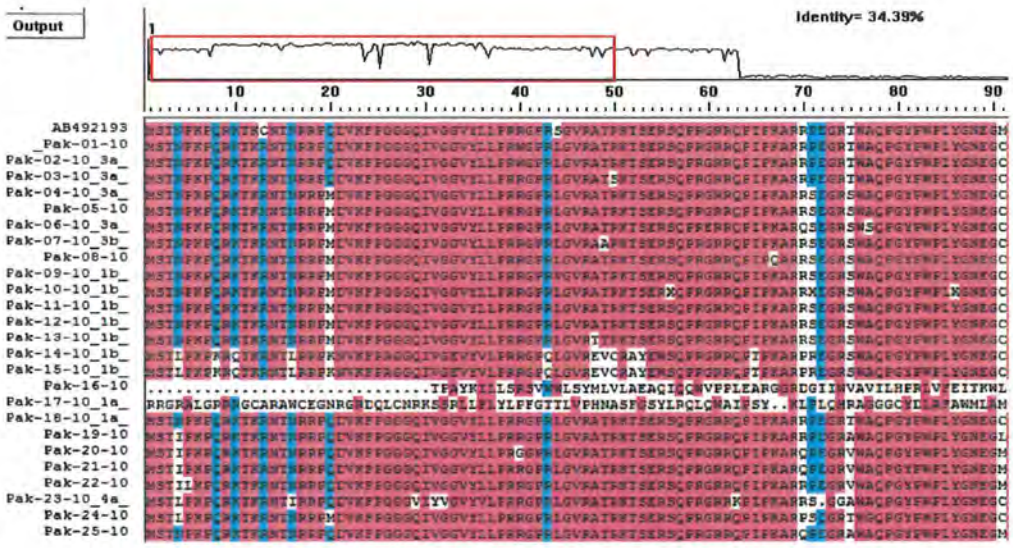


Figure 3.3.9: Representative output of DNAman. Amino acid substitution in the study sequences in comparison to the reference sequences obtained from DNAman.

3.3.8 siRNA PREDICTION FOR 5′ UTR

The siRNA were predicted using siDirect against 75 HCV samples of the non-responders to convential therapy and were resolved in the present study (Table 3.3.4). it was observed that targeted site to design siRNA are same against various genotypes describing the same domain at same position indicating that RNA and DNA binding domain is conserved in this region in those genotypes, so single siRNA can be used against various genotypes and inhibit HCV RNA synthesis. In addition efficient and safe delivery methods are required to suppress HCV replication in infected cells. Here it can be assumed that computationally designed siRNA can inhibit replication of HCV inside the cells recommends that this approach of RNA-targeting might deliver an actual therapeutic option against HCV infection, especially at the optimal site within the conserved 5'UTR. Nevertheless, the presently used approach to design siRNA is just a basic computational approach and only defined the targeted sites in the 5′ UTR sequence.

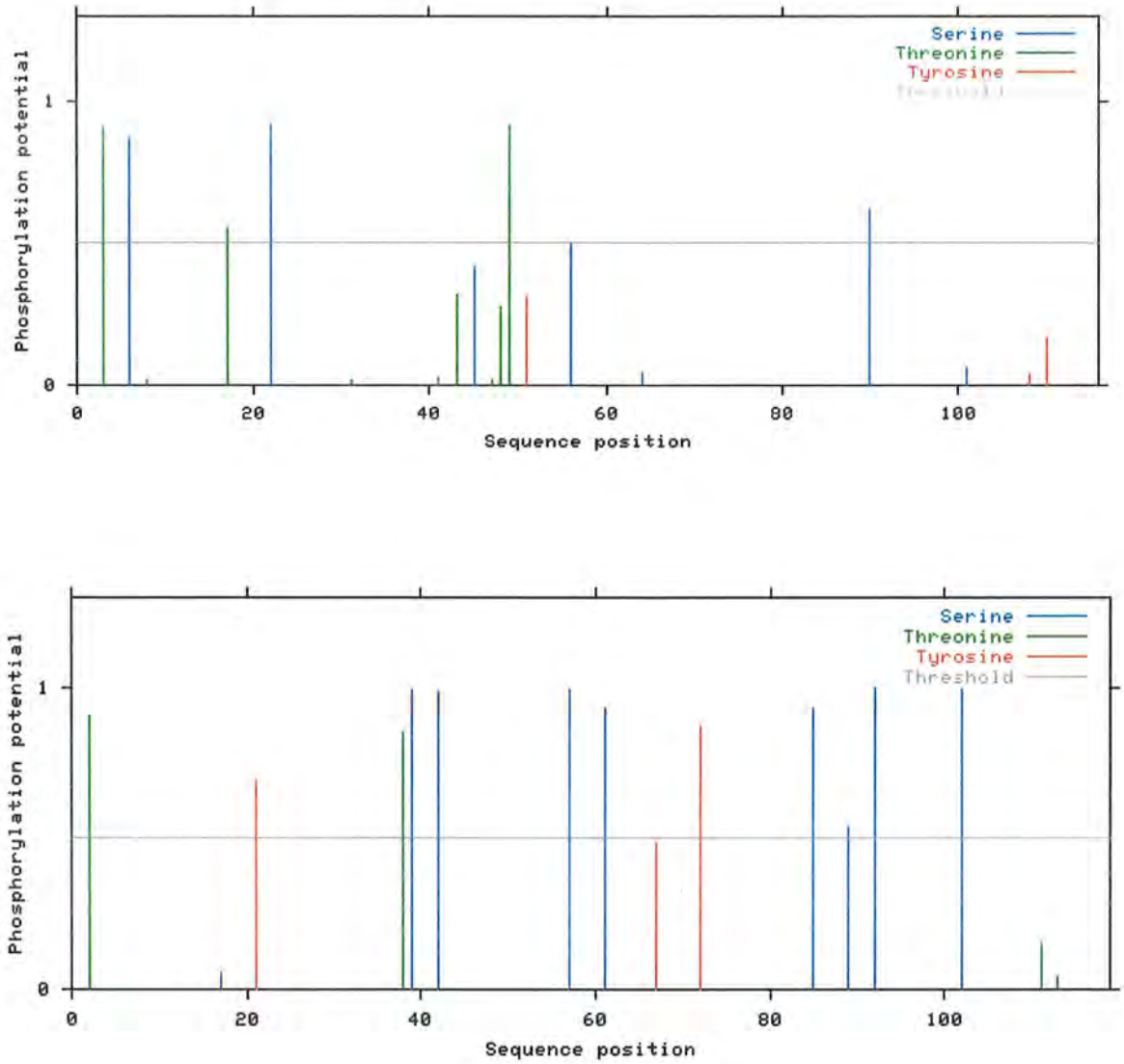
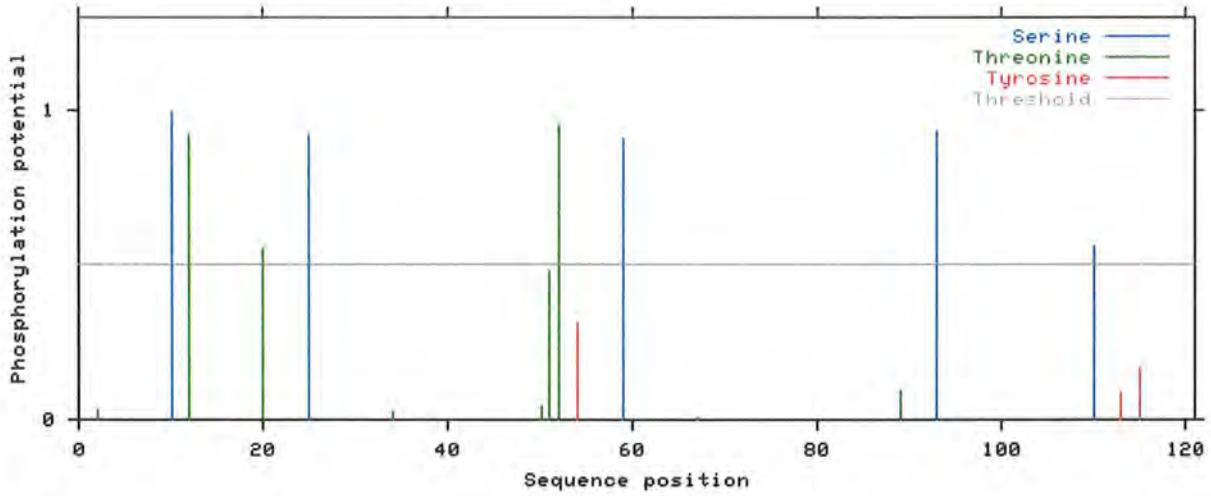
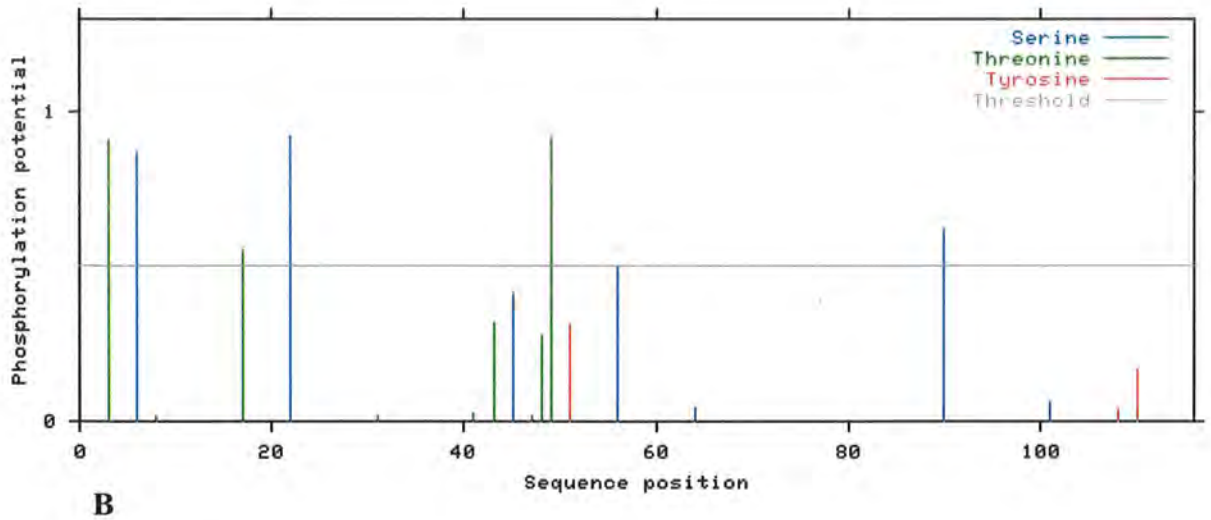


Figure 3.3.10: Output of predicted phosphorylation sites in the core protein sequences of FASTA aligned 75 sequences each in both treatment groups (A) group 1 (with SVR) and (B) group 2 (non-responders).($P < 0.002$).

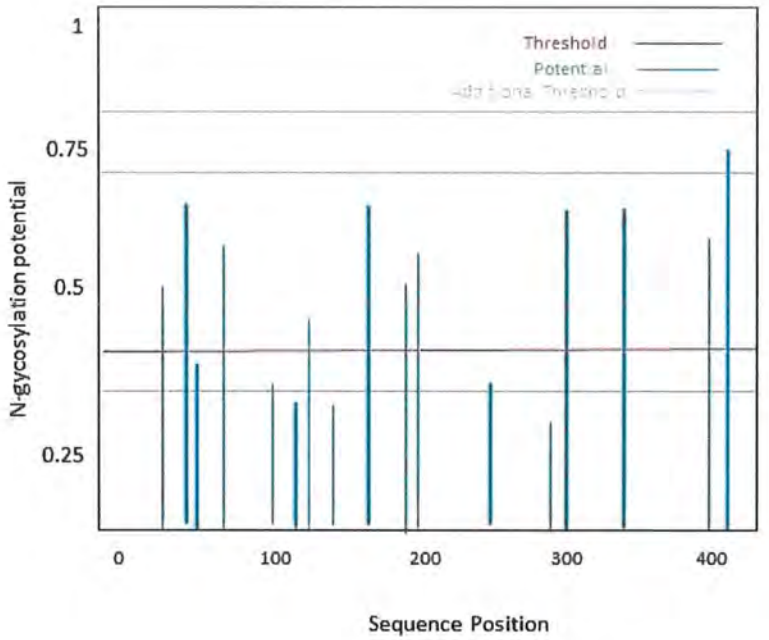


A



B

Figure 3.3.11: Output of predicted phosphorylation sites in NS5B protein sequences of FASTA aligned 75 sequences each in both treatment groups (A) group 1 (with SVR) and (B) group 2 (non-responders). ($P < 0.63$)



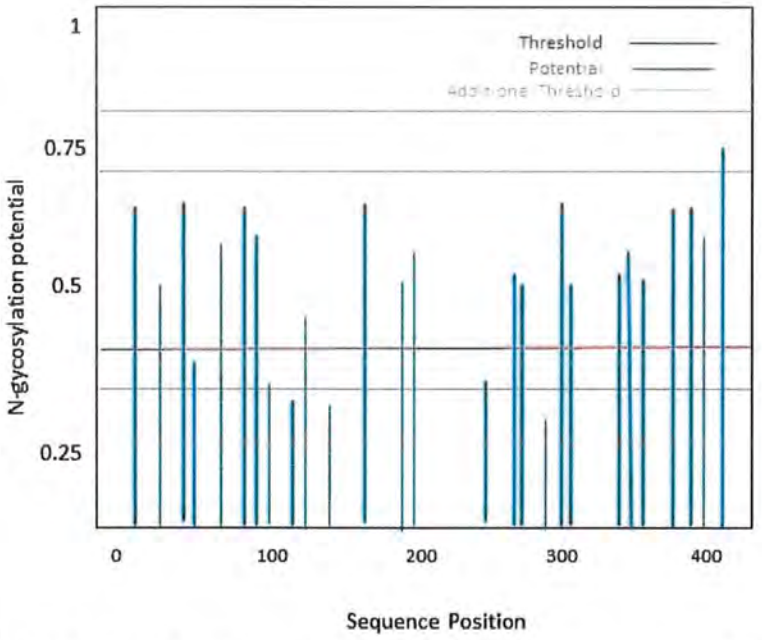


Figure 3.3.12: Output of predicted N-linked glycosylation sitesin FASTA aligned 75 sequences each of core protein in both treatment groups (A) group 1 (with SVR)and (B) group 2 (non-responders). ($P < 0.003$)

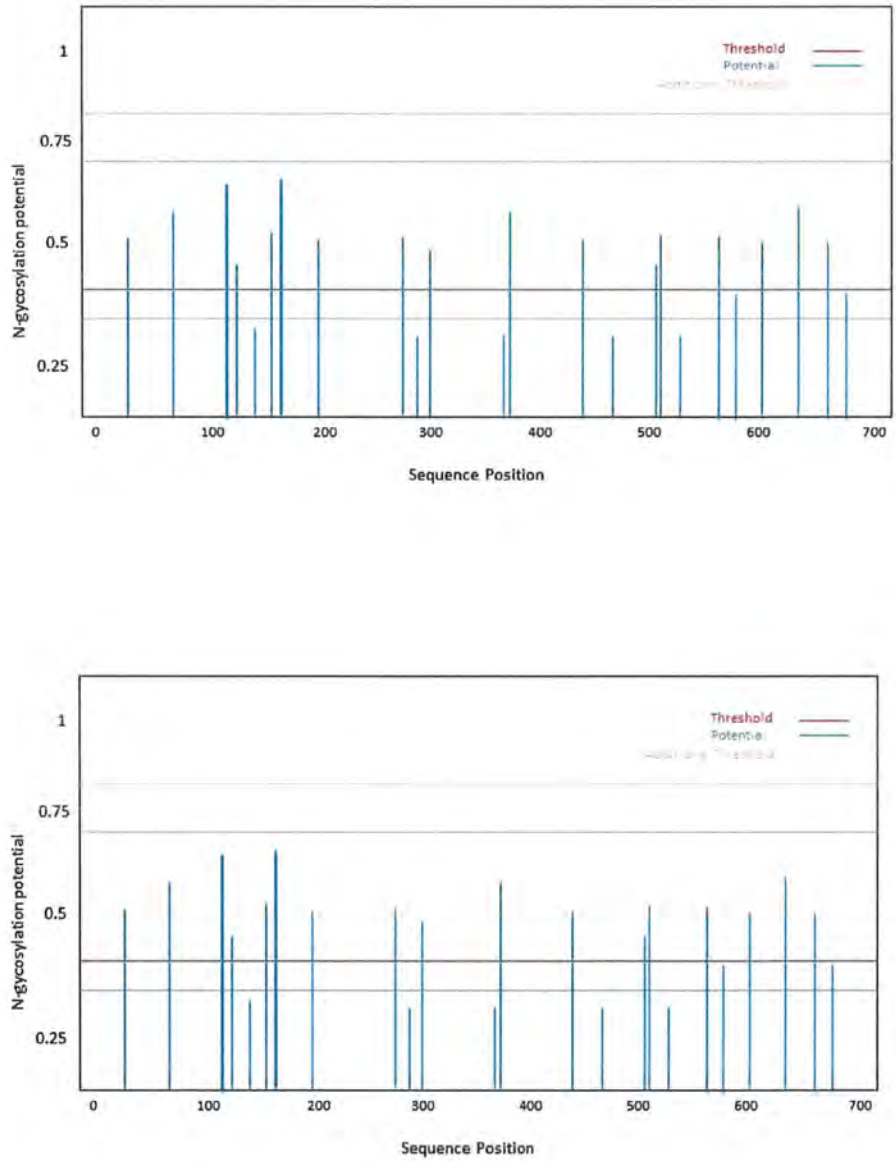


Figure 3.3.13: Output of predicted N-glycosylation sites in FASTA aligned 75 sequences each of NS5B protein in both treatment groups (A) group 1 (with SVR) and (B) group 2 (non-responders).

Table 3.3.4: Predicted siRNA against the 5′ UTR sequences of group 2 (non-responders) of present studied resolved HCV samples.

Sample ID	HCV genotype	Target Position	Target Sequence	siRNA sequence
Pak-01-10 (G2)	3a	24-46	CCGCTCAATACCCAGAAATTTG G	AAAUUUCUGGGUAUUGAGCGG GCUCAAUACCCAGAAAUUUGG
Pak-02-10 (G2)	3a	122-134	CCGCTCAATGCCCGGAAATTTG G	AAAUUUCCGGGCAUUGAGCGG GCUCAAUGCCCGGAAAUUUGG
Pak-03-10 (G2)	3a	178-200	ACGAATCCTAAACCTCAAAGAA A	UCUUUGAGGUUUAGGAUUCGU GAAUCCUAAACCUCAAAGAAA
Pak-04-10 (G2)	3a	149-171	TGCCCATTAATCACTAAACGAG T	UCGUUUAGUGAUUAAUGGGCA CCCAUUAAUCACUAAACGAGU
Pak-05-10 (G2)	3a	112-134	CCGCTCAATGCCCGGAAATTTG G	AAAUUUCCGGGCAUUGAGCGG GCUCAAUGCCCGGAAAUUUGG
Pak-06-10 (G2)	3a	124-146	CCGCTCAATGCCCGGAAATTTG G	AAAUUUCCGGGCAUUGAGCGG GCUCAAUGCCCGGAAAUUUGG
Pak-07-10 (G2)	3b	185-207	TTCCTGAAATGTGAAAAAAAAT G	UUUUUUUUCACAUUUCAGGAA CCUGAAAUGUGAAAAAAAAUG
Pak-08-10 (G2)	3b	43-65	CCGCTCAATGCCCGGAAATTTG G	AAAUUUCCGGGCAUUGAGCGG GCUCAAUGCCCGGAAAUUUGG
Pak-09-10 (G2)	1b	105-127	CCGCTCAATACCCAGAAATTTG G	AAAUUUCUGGGUAUUGAGCGG GCUCAAUACCCAGAAAUUUGG
Pak-10-10 (G2)	1b	43-65	CCGCTCAATGCCCGGAAATTTG G	AAAUUUCCGGGCAUUGAGCGG GCUCAAUGCCCGGAAAUUUGG
Pak-11-10 (G2)	1b	76-98	CCGCTCAATGCCCGGAAATTTG G	AAAUUUCCGGGCAUUGAGCGG GCUCAAUGCCCGGAAAUUUGG
Pak-12-10 (G2)	1b	149-171	TGCCCATTAATCACTAAACGAG T	AAAUUUCUGGGUAUUGAGCGG GCUCAAUACCCAGAAAUUUGG
Pak-13-10 (G2)	1b	145-167	CCGCTCAATGCCCGGAAATTTG G	AAAUUUCCGGGCAUUGAGCGG GCUCAAUGCCCGGAAAUUUGG
Pak-14-10 (G2)	1b	121-143	CCGCTCAATGCCCGGAAATTTG G	AAAUUUCCGGGCAUUGAGCGG GCUCAAUGCCCGGAAAUUUGG
Pak-15-10 (G2)	1b	146-168	CCGCTCAATGCCCGGAAATTTG G	AAAUUUCCGGGCAUUGAGCGG GCUCAAUGCCCGGAAAUUUGG
Pak-16-10 (G2)	1a	218-240	CCGCTCAATGCCCGGAAATTTG G	AAAUUUCCGGGCAUUGAGCGG GCUCAAUGCCCGGAAAUUUGG
Pak-17-10 (G2)	1a	111-134	CCGCTCAATACCCAGAAATTTG G	AAAUUUCUGGGUAUUGAGCGG GCUCAAUACCCAGAAAUUUGG
Pak-18-10 (G2)	1a	110-132	CCGCTCAATGCCCGGAAATTTG G	AAAUUUCCGGGCAUUGAGCGG GCUCAAUGCCCGGAAAUUUGG
Pak-19-10 (G2)	1a	124-146	CCGCTCAATGCCCGGAAATTTG G	AAAUUUCCGGGCAUUGAGCGG GCUCAAUGCCCGGAAAUUUGG
Pak-20-10 (G2)	Unresolved	214-236	CCGCTCAATACCCAGAAATTTG G	AAAUUUCUGGGUAUUGAGCGG GCUCAAUACCCAGAAAUUUGG
Pak-21-10 (G2)	1a	112-134	CCGCTCAATACCCAGAAATTTG G	AAAUUUCUGGGUAUUGAGCGG GCUCAAUACCCAGAAAUUUGG
Pak-22-10 (G2)	4a	209-231	GACGCTATATGCGTGAAAAGTC T	ACUUUUCACGCAUAUAGCGUC CGCUAUAUGCGUGAAAAGUCU
Pak-23-10 (G2)	4a	101-123	GGGCAAGAAATAAATTCTTGTG T	ACAAGAAUUUAUUUCUUGCCC GCAAGAAAUAAAUUCUUGUGU
Pak-24-10 (G2)	4a	24-46	CCGCTCAATACCCGGAAATTTG G	AAAUUUCCGGGUAUUGAGCGG GCUCAAUACCCGGAAAUUUGG
Pak-25-10 (G2)	4a	112-134	CCGCTCAATGCCCGGAAATTTG G	AAAUUUCUGGGUAUUGAGCGG GCUCAAUACCCAGAAAUUUGG

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Pak-26-10 (G2)	4a	208-230	CCGCTCAATACCAAGAAATTTGG	AAAUUUCUUGGUAUUGAGCGG GCUCAAUACCAAGAAAUUUGG
Pak-27-10 (G2)	4a	213-235	CCGCTCAATACCCAGAAATTTGG	AAAUUUCUGGGUAUUGAGCGG GCUCAAUACCCAGAAAUUUGG
Pak-28-10 (G2)	4c	24-46	CCGCTCAATGCCCGGAAATTTGG	AAAUUUCCGGGCAUUGAGCGG GCUCAAUGCCCGGAAAUUUGG
Pak-29-10 (G2)	4c	163-185	CCGCTCAATACCCAGAAATTTGG	AAAUUUCCGGGCAUUGAGCGG GCUCAAUGCCCGGAAAUUUGG
Pak-30-10 (G2)	4c	124-146	CCGCTCAATGCCCGGAAATTTGG	AAAUUUCUGGGUAUUGAGCGG GCUCAAUACCCAGAAAUUUGG
Pak-31-10 (G2)	5a	112-134	CCGCTCAATGCCCGGAAATTTGG	UCGUUUAGUGAUUAAUGGGCA CCCAUUAAUCACUAAACGAGU
Pak-32-10 (G2)	5a	122-144	CCGCTCAATGCCCGGAAATTTGG	AAAUUUCCGGGCAUUGAGCGG GCUCAAUGCCCGGAAAUUUGG
Pak-33-10 (G2)	5a	149-171	TGCCCATTAATCACTAAACGAGT	UCGUUUAGUGAUUAAUGGGCA CCCAUUAAUCACUAAACGAGU
Pak-34-10 (G2)	5a	124-146	CCGCTCAATGCCCGGAAATTTGG	UUACACAAGAAUUUAUUUCUU GAAAUAAAUUCUUGUGUAAUC
Pak-35-10 (G2)	6q	24-46	CCGCTCAATACCCAGAAATTTGG	AAAUUUCUGGGUAUUGAGCGG GCUCAAUACCCAGAAAUUUGG
Pak-36-10 (G2)	6e	122-144	CCGCTCAATGCCCGGAAATTTGG	AAAUUUCCGGGCAUUGAGCGG GCUCAAUGCCCGGAAAUUUGG
Pak-37-10 (G2)	4b	112-134	CCGCTCAATGCCCGGAAATTTGG	AAAUUUCCGGGCAUUGAGCGG GCUCAAUGCCCGGAAAUUUGG
Pak-38-10 (G2)	4b	163-185	TGCCCATTAATCACTAAACGAGT	UCGUUUAGUGAUUAAUGGGCA CCCAUUAAUCACUAAACGAGU
Pak-39-10 (G2)	4b	112-134	CCGCTCAATGCCCGGAAATTTGG	UUACACAAGAAUUUAUUUCUU GAAAUAAAUUCUUGUGUAAUC
Pak-40-10 (G2)	4b	122-144	CCGCTCAATGCCCGGAAATTTGG	AAAUUUCCGGGCAUUGAGCGG GCUCAAUGCCCGGAAAUUUGG
Pak-41-10 (G2)	Unresolved	209-231	GACGCTATATGCGTGAAAAGTCT	AAAUUCCGGGCAUUGAGCGG GCUCAAUGCCCGGAAAUUUGG
Pak-42-10 (G2)	2a	124-146	CCGCTCAATGCCCGGAAATTTGG	AAAUUUCUGGGUAUUGAGCGG GCUCAAUACCCAGAAAUUUGG
Pak-43-10 (G2)	2a	122-144	CCGCTCAATGCCCGGAAATTTGG	AAAUUUCCGGGCAUUGAGCGG GCUCAAUGCCCGGAAAUUUGG
Pak-44-10 (G2)	2c	209-231	GACGCTATATGCGTGAAAAGTCT	AAAUUUCCGGGCAUUGAGCGG GCUCAAUGCCCGGAAAUUUGG
Pak-45-10 (G2)	2c	124-146	CCGCTCAATGCCCGGAAATTTGG	AAAUUUCCGGGCAUUGAGCGG GCUCAAUGCCCGGAAAUUUGG
Pak-46-10 (G2)	2b	24-46	CCGCTCAATACCCAGAAATTTGG	AAAUUUCCGGGCAUUGAGCGG GCUCAAUGCCCGGAAAUUUGG
Pak-47-10 (G2)	2b	124-146	CCGCTCAATGCCCGGAAATTTGG	UUACACAAGAAUUUAUUUCUU GAAAUAAAUUCUUGUGUAAUC
Pak-48-10 (G2)	2b	112-134	CCGCTCAATGCCCGGAAATTTGG	AAAUUCCGGGCAUUGAGCGG GCUCAAUGCCCGGAAAUUUGG
Pak-49-10 (G2)	2b	111-134	CCGCTCAATGCCCGGAAATTTGG	AAAUUUCCGGGCAUUGAGCGG GCUCAAUGCCCGGAAAUUUGG
Pak-50-10 (G2)	2b		TGCCCATTAATCACTAAACGAGT	UUACACAAGAAUUUAUUUCUU GAAAUAAAUUCUUGUGUAAUC
Pak-51-10 (G2)	1b	149-171	TGCCCATTAATCACTAAACGAGT	UCGUUUAGUGAUUAAUGGGCA CCCAUUAAUCACUAAACGAGU
Pak-52-10 (G2)	6v	24-46	CCGCTCAATACCCAGAAATTTGG	AAAUUUCCGGGCAUUGAGCGG GCUCAAUGCCCGGAAAUUUGG

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Pak-53-10 (G2)	1c	122-144	CCGCTCAATGCCCGGAAATTTGG	AAAUUUCCGGGCAUUGAGCGG GCUCAAUGCCCGGAAAUUUGG
Pak-54-10 (G2)	1c	163-185	TGCCCATTAATCACTAAACGAGT	UUACACAAGAAUUUAUUUCUU GAAAUAAAUUCUUGUGUAAUC
Pak-55-10 (G2)	1c	24-46	CCGCTCAATACCCAGAAATTTGG	ACAAGAAUUUAUUUCUUGCCC GCAAGAAAUAAAUUCUUGUGU
Pak-56-10 (G2)	1c	24-46	CCGCTCAATACCCAGAAATTTGG	AAAUUCCGGGCAUUGAGCGG GCUCAAUGCCCGGAAAUUUGG
Pak-57-10 (G2)	3c	122-144	CCGCTCAATGCCCGGAAATTTGG	UUACACAAGAAUUUAUUUCUU GAAAUAAAUUCUUGUGUAAUC
Pak-58-10 (G2)	3c	112-134	CCGCTCAATGCCCGGAAATTTGG	AAAUUCCGGGCAUUGAGCGG GCUCAAUGCCCGGAAAUUUGG
Pak-59-10 (G2)	3c	163-185	TGCCCATTAATCACTAAACGAGT	UUACACAAGAAUUUAUUUCUU GAAAUAAAUUCUUGUGUAAUC
Pak-60-10 (G2)	5b	112-134	CCGCTCAATGCCCGGAAATTTGG	AAAUUUCCGGGCAUUGAGCGG GCUCAAUGCCCGGAAAUUUGG
Pak-61-10 (G2)	5b	112-134	CCGCTCAATGCCCGGAAATTTGG	AAAUUCCGGGCAUUGAGCGG GCUCAAUGCCCGGAAAUUUGG
Pak-62-10 (G2)	5b	111-134	CCGCTCAATGCCCGGAAATTTGG	AAAUUUCUGGGUAUUGAGCGG GCUCAAUACCCAGAAAUUUGG
Pak-63-10 (G2)	5b	124-146	CCGCTCAATGCCCGGAAATTTGG	AAAUUUCCGGGCAUUGAGCGG GCUCAAUGCCCGGAAAUUUGG
Pak-64-10 (G2)	6a	24-46	CCGCTCAATGCCCGGAAATTTGG	AAAUUUCCGGGCAUUGAGCGG GCUCAAUGCCCGGAAAUUUGG
Pak-65-10 (G2)	6a	2-24	GCCAAAATTTCTGGGTATTGAGC	UCAAUACCCAGAAAUUUUGGC CAAAAUUUCUGGGUAUUGAGC
Pak-35-10 (G2)	6a	111-134	TGCCCATTAATCACTAAACGAGT	AAAUUUCCGGGCAUUGAGCGG GCUCAAUGCCCGGAAAUUUGG
Pak-66-10 (G2)	6a	112-134	CCGCTCAATGCCCGGAAATTTGG	AAAUUCCGGGCAUUGAGCGG GCUCAAUGCCCGGAAAUUUGG
Pak-67-10 (G2)	6a	115-137	CCGCTCAATACCCAGCAATTTGG	AAAUUGCUGGGUAUUGAGCGG GCUCAAUACCCAGCAAUUUGG
Pak-68-10 (G2)	3a	111-133	CCGCTCAATGCCCGGAAATTTGG	AAAUUUCCGGGCAUUGAGCGG GCUCAAUGCCCGGAAAUUUGG
Pak-69-10 (G2)	3a	111-134	CCGCTCAATGCCCGGAAATTTGG	ACUUUUCACGCAUAUAGCGUC CGCUAUAUGCGUGAAAAGUCU
Pak-70-10 (G2)	3a	110-132	CCGCTCAATACCCAGAAATTTGG	AAAUUUCUGGGUAUUGAGCGG GCUCAAUACCCAGAAAUUUGG
Pak-71-10 (G2)	3a	112-134	CCGCTCAATGCCCGGAAATTTGG	AAAUUUCCGGGUAUUGAGCGG GCUCAAUACCCGGAAAUUUGG
Pak-72-10 (G2)	3a	163-185	CCGCTCAATGCCCGGAAATTTGG	ACUUUUCACGCAUAUAGCGUC CGCUAUAUGCGUGAAAAGUCU
Pak-73-10 (G2)	Unresolved	112-134	CCGCTCAATGCCCGGAAATTTGG	ACAAGAAUUUAUUUCUUGCCC GCAAGAAAUAAAUUCUUGUGU
Pak-74-10 (G2)	Unresolved	163-185	CCGCTCAATGCCCGGAAATTTGG	AAAUUCCGGGCAUUGAGCGG GCUCAAUGCCCGGAAAUUUGG
Pak-75-10 (G2)	Unresolved	163-185	TGCCCATTAATCACTAAACGAGT	UUACACAAGAAUUUAUUUCUU GAAAUAAAUUCUUGUGUAAUC

DISCUSSION

4.1 SEROEPIDEMIOLOGY OF HCV POSITIVE PATIENTS IN RESPONSE TO THE TREATMENT

In the present study high ratio of the HCV positive males was observed as compared to females (Figure 3.1.1). Majority of the previous studies reported from all over the world including Pakistan, where the number of male patients was greater than females (Kim et al., 1998; Khan et al., 2003; Muhammad and Jan, 2005 and Khan and Zarif, 2006). It is already known that progression of HCV infection is worse in males than in females (Bissell, 1999 and Seeff, 1997). Independent of alcohol intake, females have twofold lesser progression rate to fibrosis compared with males (Poynard et al., 1997). In males higher rate of HCV prevalence is possibly due to exposure to various risk factors. This trend might be due to the fact that males particularly from Pakistani social setup are more exposed to the HCV risk factors i.e blood transfusions, dental procedures, barber shears, needle sick injuries and tattooing as compared to female patients. According to our cultural environment there is trivial exposure of females to some of the risk factors e.g. tattooing, injection drug use, barbers etc. Moreover our cultural trait associates the higher prevalence in males.

4.1.1 Natural History of the HCV for the Studied Cohort

There are two broad categories of HCV infection, acute and chronic. The term acute is used for the infection that persists for six months or less time period, while chronic HCV infection is marked by the persistence of the viral RNA in the blood and hepatocytes for at least six months after the onset of infection (Chen and Morgan, 2006). In the current study the selected cohort of patients constituted of 80 % chronic cases of HCV and only 20 % acute infection (Figure 3.1.2). These results are in accordance with the ones previously reported by Al-Moslih and Al-Huraibi (2001) from Yamen, where the majority of the reported cases were chronic and the number of acute cases was 19.4 %. Major reason supposed to be involved in this chronicity pattern is that the HCV infection is asymptomatic in 75-80 % of the cases. It has been studied that only 20-25 % of the patients report to the hospitals with some presenting complaints (Herrine, 2002). However, in Pakistan a common man does not prefer to visit physician for routine checkup, which might leads to chronicity of disease.

There were no apparent gender differences in the rate of chronicity in hepatitis C infection. Majority of the considered cases whether males or females were equally suffering from the chronicity of the disease (Figure 3.1.2). Similar observations were reported from other studies around the world, such as chronicity was related to age and the estimated rate was 30 % and 76 % in subjects' ≤ 20 and ≥ 20 years of age, respectively (Alter et al., 1999 and Bellentani and Tiribelli, 2001). Acording to a report among three to four million newly infected people each year, 170 million people are chronically infected and at risk of developing liver disease including cirrhosis and liver cancer, and 350,000 deaths occur each year due to all HCV-related causes around the globe (Perz et al., 2006).

4.1.2 Seroepidemiology of HCV

Among the other blood borne pathogens, HCV is the most destructive one and cause a major health issue now a day worldwide (Alter, 2007 and Asselah et al., 2010). The disease severity progresses from asymptomatic condition to HCC through chronic infection and cirrhosis (Seeff, 1997 and Marcellin et al., 2002). Number of patients presenting long term complications of this infection is expected to increase in next 20 years as a protective vaccine against HCV does not yet exist and the therapeutic options remained limited (Williams, 2006). Eradication of virus is the main objective of treatment against HCV defined by SVR. It has been reported in different studies that IFN monotherapy showed increase in SVR rate from approximately 6–12 % to 38–42 % while with usual IFN and RBV combination it increased as high as 55 % in major clinical trials (Poynard et al., 1995; Mchutchison et al., 1998; Manns et al., 2001 and Fried et al., 2002).

In a report it was observed that in more than 40 % of the patients antiviral therapy failed to control viral replication (non-responders) and even reoccurrence of the infection experienced when therapy stopped (Gao et al., 2004). Therefore, it is of great importance to get insight the mechanisms involved in non-responsiveness and further to recognize the factors that can predict the likelihood of patient's response towards treatment. Various viral and host factors are involved in non-responsiveness besides the mechanism stimulated by HCV proteins at molecular level to reduce the IFN signaling pathway. In the present study, the serum analysis for selected biochemical markers directly related with liver disease was performed for the final participants of the study and further the correlation of the serum biochemical markers

was checked with HCV viral load. The obtained results were analyzed using different statistical methods.

4.1.2.1 Determination of Viral Load

Viral load or the amount of HCV RNA in the patient's blood is thought to characterize the persistence of viral replication and clearance. This is predominantly useful for monitoring effect of anti-HCV drug therapy. It is well recognized that serum level of HCV RNA is directly related with response to IFN therapy in patients with chronic HCV infection (Davis et al., 1994 and Martinot-Peignoux et al., 1998). In the present study viral load was quantified as the predictive factor for HCV response to treatment. Patients in group 1 possess low baseline viral load that dropped significantly with the start of therapy (Figure 3.1.4). This is steady with the previous report where poor response to standard antiviral therapy was observed in patients with higher viral load in contrast to patients having lower viral load (Bell et al., 1997). The degree of HCV infection has been associated with increased viral load (Bonkovsky and Mehta, 2001).

Many investigators reported that baseline viral load has been linked with low rates of response to standard interferon therapy (Martinot-Peignoux et al., 1995; Bell et al., 1997 and Franciscus, 2006). Therefore, it is shown that along with HCV genotypes, baseline viral load also act as predictor of response to antiviral therapy (Yoshioka et al., 1992). Several studies have shown positive response to currently available antiviral therapy in patients with lower baseline viral load (< 80,0000) in comparison to high pre-treatment viral load (> 80,0000) (Hayashi et al., 1998; Dalgard et al., 2004 and Von Wagner et al., 2005). Similar findings also suggest that a decline in HCV viral load during the first 2-12 weeks of antiviral therapy is an indicative of prognostic therapeutic efficacy (Kakumu et al., 1997 and Vrolijk et al., 2004). Therefore, HCV baseline viral load, decrease of viral load during initial phase of therapy and genotype exhibited important roles in altering and improving antiviral treatment (Ahmad et al., 2010). Herein non-response to the therapy might be due to high baseline viral load, further there must be some additional host and viral factors that would be responsible for the viral response to the treatment.

4.1.2.2 Comparative Analysis of LFTs with the Antiviral Therapy

In the present study, patients in group 1 (with SVR) showed a significant decrease in serum ALT, AST, TBL, total protein and albumin with the progression of

therapy as compared to patients in group 2 (non-responders) (Table 3.1.2; Figure 3.1.5 and 3.1.6). Two enzymes ALT and AST are present in higher concentration in liver cells and are most commonly used as indicators of liver cell necrosis. Any damage or injury to liver cell membranes leads to the leakage of aminotransferases into the circulation (Dufour et al., 2000). The role of liver enzymes in the assessment of chronic hepatitis C remains important due to the fact that the majority of clinical indexes estimating the degree of liver fibrosis are based on liver transaminases (Sheth et al., 1998; Imbert-Bismut et al., 2001; Giannini et al., 2003; Wai et al., 2003; Forns et al., 2004 and Lackner et al., 2005). Serum ALT remained the most accessible test for monitoring the chronic hepatitis C viral infection (Tsang et al., 2008). Various previous studies are in accordance with the present results that showed the normalization of the LFTs levels with the therapy received (Hoofnagle, 1997; Dufour et al., 2000 and Kim et al., 2008).

The presence of underlying chronic hepatitis C significantly increased the risk of elevations in liver enzyme levels during antiviral therapy (Bonfanti et al., 2001; Sulkowski et al., 2002 and Servoss et al., 2006). Liver damage, membranoproliferative intraoperative hypotension, intraoperative blood loss, liver glomerulonephritis associated with HCV infection significantly correlated with liver enzyme infection elevations (Hsu et al., 1991). It has also been reported that being the biomarker for inflammation of liver, serum ALT level is a widely used clinical practice (Kim et al., 2008). Moreover, liver cirrhosis and HCC followed by liver tumor and metastases by active or nonactive HCV or HBV is shown to be evidently associated with bilirubin levels (Raymond et al., 2002).

4.1.2.2.1 Association of Liver Profile with HCV Viral Load

Overall the patients in group 1 (with SVR) showed direct correlation of liver profile and their viral load while a linear reduction in serum ALT, AST and total protein was observed in non-responders (group 2) (Table 3.1.3; Figure 3.1.7 and 3.1.8). Therefore, it can be assumed that presence of viral RNA in the serum might interfere the immune response of the patients leading to non-responsiveness towards therapy and the serum markers also remain unaffected.

Furthermore, weak correlation of serum viral loads with ALT in the present study is in promise with the reports of Azzari et al. (2000) and Murakami et al. (2004) indicating that the viral load is independent of ALT activity in HCV positive patients.

In another investigation, Zechini et al. (2004) found a relation between HCV viral load and AST. However, the correlation among HCV genotypes with serum markers and viral load and their relation with severity of disease and receptivity to interferon treatment remains controversial (de Moliner et al., 1998 and Al-Khurri et al., 2009).

4.1.2.3 Comparative Analysis of Lipid Profile in All Studied Cohorts

Liver is the primary site of lipoprotein formation and clearance; in addition it is involved in many stages of lipid metabolism and its transport. Therefore in case of severe liver disease, a profound disturbance occurs in lipid metabolism and affects it in a variety of ways. There is marked elevation of free cholesterol and phospholipids in obstructive liver disease. In acute hepatocellular disease such as alcoholic or viral hepatitis, there is a cholestatic phase and similar changes may be seen e.g. increased cholesterol and phospholipid levels (Miller, 1990).

It was also observed from the present data that the patients who respond to therapy (group 1) showed improvement in their lipid profile and normalization of serum concentrations of LDL, HDL, TGs, and serum total cholesterol occurred with the progression of the therapy. While the patients who failed to respond therapy (group 2) did not show any significant change in their lipid profile (Table 3.1.4; Figure 3.1.9 and 3.1.10). Previously, Malavazi et al. (2004), Ooi et al. (2005) and Ramcharran et al. (2011), also found associations between serum lipid profiles and both HCV level plus liver disease severity and reported an association between viral infection and abnormal lipid metabolism. Moreover, marked lipid abnormalities were also found in patients suffering from HCV and HIV coinfection (Bedimo et al., 2006). Furthermore, Fabris et al. (1997) and Serfaty et al. (2001) observed the association between chronic HCV and lipid metabolism.

Lipids are vital component of biological membranes, exist as free molecules and act as metabolic regulators by controlling homeostasis and cellular function (Chiang et al., 2005) and also play a dynamic role in lipid metabolism. Therefore, several important lipid–HCV interactions have also been found (Diaz et al., 2006). Hence the present study showed the role and diagnostic value of lipid profile in HCV infection progression. Antiviral therapy intake might modulate the host immune system that further effect to normalize the lipid profile of the patients.

4.1.2.3.1 Correlation of Serum Lipid Profile with HCV Viremia

Association of HCV genotypes with change in host's normal lipid profile has been reported by many investigators (Maggi et al., 1996; Fabris et al., 1997 and Moriya et al., 2003), however only few reports showed the correlation between serum HCV viral load and host lipid profiles (Siagris et al., 2006 and Hsu et al., 2009). In the present study, overall weak and direct association between lipid profile and viral load was observed (Table 3.1.5; Figure 3.1.11 and 3.1.12). Similar to the present data, an earlier investigation reported the association of HCV infection and abnormal lipid metabolism in the liver (Malavazi et al., 2004).

Therefore, it can be suggested that HCV and its life cycle may be related to serum lipids. In earlier studies, lowering of LDL, HDL and TCOL levels were observed in patients with chronic HCV as compared to people without this infection (Jarmay et al., 2005; Moritani et al., 2005 and Marzouk et al., 2007). Data is also available for both lower and higher levels of TG associated with HCV infection (Targher et al., 2007), the reverse trend of the associations perhaps reflecting the influence of various host factors. Currently, substantial direct association between HCV RNA titers and levels of TG has been observed, which is consistent with the findings from *in vitro* work proposing that hepatocytes release the HCV into the serum complexed with VLDL (Perlemuter et al., 2002; Nielsen et al., 2006; Gastaminza et al., 2007; Huang et al., 2007 and Ye, 2007).

4.1.2.4 Comparative Analysis of Selected Blood Parameters among all the Studied Groups

Synthesis of coagulation proteins and haemopoiesis involve the central role of liver thus many haematological abnormalities are related with liver disease because of the unique portal circulation and its synthetic (clotting factors, thrombopoietin) plus immune functions. Hematological abnormalities are result of primary liver problems like cirrhosis and primary hematological diseases can in turn affect the liver and its functioning. Various kinds of hematological abnormalities have been known to occur in HCV infection and other liver diseases.

It has been observed that directly infected megakaryocytes resulted in abnormal platelet production and megakaryocytic apoptosis (Sakaguchi et al., 1991 and Ballem et al., 1992). Evident cirrhosis and advanced liver fibrosis, deficiency of hepatic-derived thrombopoietin (Afdhal et al., 2008), direct cytopathic involvement of megakaryocytes and platelets (Bordin et al., 1995 and de Almeida et al., 2004) and

HCV-associated immunoglobulins that can prompt thrombocytopenia through an immunological mechanism comparable the system operating in immune thrombocytopenic purpura (Aref et al., 2009) are the outcome of thrombocytopenia in chronic HCV infection (Wang et al., 2004).

In the current study, all the three parameters (PTT, Hb and PLT) showed progressive change as the therapy proceeded in group 1 as compared to group 2 (Table 3.1.6 and Figure 3.1.13). The PTT is influenced by the activity of factors I, II, V, VII, and X, all of which are synthesized in the liver, and three of which (II, VII, and X) require vitamin K. The PTT may be prolonged in the absence of vitamin K, because vitamin K cannot be absorbed, or because it is not synthesized as a result of hepatic cell injury. To recover the vitamin K deficiency, the most applicable way is a trial of vitamin K injections (Johnston, 1999). Thrombogenesis is directly affected by either a direct suppressive effect on the bone marrow or it might have a direct effect on the megakaryocytes, leading to decreased platelet production (Espanol et al., 2000). Similar to the current findings regarding another important parameter Hb, in a previous study it has been reported that chronic hepatitis C infection is associated with a higher Hb level (Sahin et al., 2003).

4.1.2.4.1 Correlation of HCV Viremia with Hematological Indices

Nonsignificant correlation of Hb, PTT and PLT with HCV viral load was observed in the patients of both groups (1 and 2) (Table 3.1.7; Figure 3.1.14 and 3.1.15). A study revealed that disease severity is independent of serum markers (Ahmad et al., 2011). Moreover, a decline in both platelet number and function was observed in chronic liver disease (Senzolo et al., 2007), and decrease in platelet count was shown with the progression of the disease especially in the incident of HCV infection. Decrease in platelet count in chronic liver disease might be because of splenic platelet sequestration due to hypersplenism (Afdhal et al., 2008) or a reduced activity or level of thrombopoietin (Eissa et al., 2008), which is a hematopoietic factor for thrombocytes production by mature hepatocytes (Shimada, 1995). Thrombocytopenia in chronic HCV infection involves the suppression of bone marrow by HCV itself or immune-mediated destruction of platelets through production of anti-platelet antibody or formation of immune complexes (Weksler, 2007). As indicated in an earlier report that most of the coagulation factors are

synthesized only in the liver (Peck-Radosavljevic, 2007) so, abnormal coagulation and tendency to bleed profusely might be the result of liver damage.

4.2 MOLECULAR CHARACTERIZATION OF HCV

The serum samples of the patients from both the treatment groups were further characterized at genetic level. Earlier the association of HCV genotyping with epidemiological studies, clinical management of the infection, response rates to antiviral treatment and vaccine development and has been reported (Liew et al., 2004). Generally, HCV has been divided into six major genotypes that can be further divided into several subtypes from A to L (Simmonds, 2001). These genotypes exhibit 30 % difference in their amino acid sequence (Simmonds et al., 2005) and are the strongest prognostic factor for SVR since patients with different HCV genotypes act differently against IFN-α therapy (Trepo, 1994 and Zein et al., 1996). Therefore in the current study, characterization of HCV genotypes in response to antiviral therapy was done on the basis of three genomic regions 5′ UTR, core, and NS5B. Moreover various analyses were performed on the obtained sequences of the concerned genomic regions.

4.2.1 Sequencing of Amplified Genomic Regions

Due to the promising clinical implications of different HCV genotypes, various reliable methods are required to categorize and characterize viral genomes from primary patient isolates. The most specific method for determining genotype implicates the phylogenetic analysis following by direct sequencing of a particular PCR-amplified region of the viral genome acquired from a patient sample. Several genomic regions of the HCV genome includes NS5, core, E1 and 5′ UTR have been used for viral genotyping using this method (Zein, 2000). Sequencing, often of multiple regions of the viral genome, in combination with the generation of phylogenetic trees, results in an accurate classification of primary patient isolates (Simmonds et al., 1994 and Simmonds et al., 1996). Furthermore, sequencing methods provide complete information about the amplified region including the detection of polymorphisms within the viral genome that may or may not have clinical relevance (Kao, 2002). Although all of these methods are able to identify the major groups of HCV, only direct nucleotide sequencing is efficient in discriminating among subtypes, detecting known mutations and elucidating new polymorphisms that may have clinical implications (Zein, 2000 and Dev et al., 2002).

Sequencing procedures are very important as they provide direct glimpse as far as viral genotypes are concerned, secondly sequencing can be the basis for molecular phylogeny. It is a very important tool used in recent years to find the roots of transmission of HCV, treatment responses, mutation sites, secondary protein structures etc. Here in the current study three genomic regions were targeted for sequencing for both the treatment groups (Figure 3.2.4).

4.2.2 Blast Analysis

Blast analysis was performed for all the sequences. The objective was to resolve the genotypes of the sequenced samples. The results of blast analysis provided high sequence similarity scores between the analyzed sequences and already reported sequences of 5' UTR, core and NS5B. Percentage similarity of sequenced sample with already reported sequences from blast is mentioned in table 3.2.1.

Different web sites used for blast are NCBI http://blast.ncbi./nlm.nih./gov/Blast.cgi/ and EMBL http://www.ebi./ ac.uk/Tools// blast2// nucleotide.html. Due to the variations and mutations in HCV RNA, six major and numerous subtypes of the HCV have been recognized (Moatter et al., 2002 and Poynard et al., 2003). These genotypes are important predictors for duration of antiviral therapy, choice of interferon and probability of SVR (Lauer and Walker et al., 2001; Poynard et al., 2003 and Idrees et al., 2008). The expected genotypes from the obtained sequences of the present study are discussed below:

4.2.2.1 Genotype 3

Number of patients exhibiting genotype 3 (a and b) is more in group 1 (SVR) (Table 3.2.1 and Figure 3.2.5) as compared to group 2 (non-responders) (Figure 3.2.6 and Table 3.2.2) describing the positive response of genotype 3 to antiviral therapy. Previously, it was also established that genotype 3 is associated with short treatment duration and also with less cost and side effects (Abbas et al., 2009). Several authors described HCV genotype 3 (a and b) as the most prevalent genotype in Pakistan (Moatter et al., 2002; Khokhar et al., 2003; Afridi et al., 2009; Akbar et al., 2009 and Bostan, 2010). Moreover, different reports revealed that the number of patients with HCV genotype 3 are increasing with time in Pakistani population (Iqbal et al, 2007; Idrees and Riazuddin, 2008; Idrees et al., 2008; Pawlotsky, 2009 and Bostan, 2010). Hence an increased rate of HCV genotype 3 in Pakistan is a good anticipation for treatment as well as control of HCV infection (Qazi et al., 2006 and Ahmad et al.,

2010). Furthermore, the prevalence of this genotype in Pakistani population established its predominance in the neighboring countries i.e. India (Qazi et al., 2006; Ijaz et al., 2008 and Husain et al., 2009), Iran (Ziyaeyan et al., 2011), Bangladesh (Qazi et al., 2006 and Inamullah et al., 2011) and China (Husain et al., 2009).

4.2.2.2 Genotype 1

Present study showed the presence of genotype 1 in both treatment groups (Figure 3.2.5, 3.2.6 and Table 3.2.1, 3.2.2). In group 1, 10 patients exhibited the genotype 1b while among the patients in group 2, 8 were presented with 1b, 5 with 1a and 4 with 1c. Overall in our study genotype 1 is more prevalent in non-responders as compared to the patients who responded to the therapy. This is similar to the previous investigations where chronic infection with HCV genotype 1 was associated with greater resistance and lower SVR rates than other genotypes (Neumann et al., 2000; Nguyen et al., 2002; Pawlotsky, 2003; Carlsson et al., 2005 and Boulestin et al., 2006). Genotype 1 is the second highest genotype after genotype 3 in Pakistan. Genotype 1a and 3a are most prevalent in West Iran and shares a long border with a province of Pakistan, Balochistan (Asif et al., 2009 and Zarkesh-Esfahani et al., 2010). Moreover, high prevalence of genotype 3a and 1b has been reported in China (Husain et al., 2009), another neighboring country of Pakistan. Local persons travelling for trade and job from these countries might be the reason for the transfer of genotype 1 in Pakistan.

4.2.2.3 Genotype 4

The number of patients possessing genotype 4 is high in non-responders in the current study (Figure 3.2.5, 3.2.6 and Table 3.2.1, 3.2.2). This might be due to the fact that previously this genotype has been considered difficult to treat because initial clinical trials using conventional IFN-α monotherapy produced limited effect (Zylberberg et al., 2000 and Kamal et al., 2011). Moreover, diversity of subtypes of genotype 4 was also observed in this group. Earlier, it has been reported that this genotype is associated with liver cirrhosis (el-Zayadi et al., 1996). In actual genotype 4 is the most important and prevalent strain of Egypt (Genovese et al., 2005), North Africa and the Middle East (Chamberlain et al., 1997 and Abdulkarim et al., 1998). Some studies from Pakistan supported the presence of genotype 4 in Pakistani population and reported prevalence is 3% (Iqbal et al., 2007) and 2.48 % (Idrees et al.,

200b). One possible reason for the existence of this genotype in Pakistan might be the neighboring country Iran where genotype 4 is a prevalent genotype which may be due to its geographical location near to Europe and Middle East (Mellor et al., 1995 and Kabir et al., 2006).

4.2.2.4 Genotype 5

Studies showed that HCV genotype 5 seems to be an easily remediable virus, with response rates harmonious with those of genotypes 2 and 3 after a 48-week course of therapy (Hnatyszyn, 2005 and Nguyen and Keeffe, 2005). This is contradictory to our study where it showed the resistance to antiviral therapy. It has been found that only non-responders showed the presence of genotype 5 while there is no observation about the presence of genotype 5 in group 1 (with SVR) (Figure 3.2.5, 3.2.6 and Table 3.2.1, 3.2.2). Genotype 5 is prevalent only in South Africa (Simmonds et al., 1993) and is very rare in Asia while Attaullah et al. (2011) compiled some reports from Pakistan which declared the presence of genotype 5 in this region.

4.2.2.5 Genotype 2

Patients in group 1 (with SVR) have not shown the prevalence of genotype 2 while non-responders exhibited the presence of genotype 2 (Figure 3.2.5, 3.2.6 and Table 3.2.1, 3.2.2). This genotype was initially reported in Africa (Mellor et al., 1995). However, according to Attaullah et al. (2011) genotype 2 is also prevalent in Pakistan. This genotype is considered as interferon responsive (Mangia and Mottola, 2012) while in the present study the patients possess the HCV genotype 2 belongs to group 2 (non- responders). Moreover, in the present study it can be suggested that patients possessing genotype 2 and 5 were more than 40 years in age and their nonresponse might be due to some other demographic characteristic or mode of viral transmission other than genotype.

4.2.2.6 Genotype 6

Only non-responders exhibited the presence of genotype 6 and occurrence of genotype 6q and 6v have been reported for the first time from Pakistan in this study (Figure 3.2.5, 3.2.6 and Table 3.2.1, 3.2.2). A few published studies suggest that genotype 6 behaves more similar to genotypes 2 and 3 in respect to treatment responses (Nguyen and Keeffe, 2005, Shepard et al., 2005 and Yuen and Lai, 2006) and responds better to therapy than genotype 1. At present, there has been no data on

the efficacy of currently FDA-approved DAAs on HCV-6 or its subtypes, but some investigational agents have been shown to induce viral suppression in small numbers of HCV genotype 6 patients (Flisiak et al., 2009 and Hezode et al., 2011). These newly identified subtypes of genotype 6 in this geographical region might own some mutation that resists the viral response towards therapy.

4.2.2.7 Untypeable genotype

In group 1 (SVR) genotypes of all the samples were resolved while among patients of group 2 (non- responders) 5 sample remained unresolved. However, based on phylogenetic trees, we can suggest their genotypes due to their close appearance with some known genotypes. Additionally to further confirm their genotype, more genomic regions can be a target or might be the whole genome. Untypability of the genotypes is caused by the mutations (McOmish et al., 1994 and Davidson et al., 1995) and these mutations may be either point mutations (Transitions and stransversions) or they may be insertions, inversions, or deletions and translocations etc. The inability of HCV to perform proof reading and its high mutation rate both have made it genetically successful according to Darwinian theory of natural selection (Stumpf and Pybus, 2002).

4.2.3 Relationship of Genotypes with Demographics of Patients

Patients in group 1 (with SVR) were ≤ 42 years in age while in group 2 the mean age of patients was more than 42 years (Table 3.2.3 and 3.3.4). Previous reports suggest that younger individuals (usually < 40 years of age) respond positively to IFN-α treatment than older persons (Reddy et al., 2009). In Pakistan, HCV is more prevalent in old age group population (Muhammad and Jan, 2005). It has been reported that in elderly population, chronic disease, immunological suppression and synchronized medications deleteriously affect response and intensify the likelihood of adverse reactions to antiviral therapy (Sezaki et al., 2009).

From patient's demographic data, it has been observed that blood or blood born products are main source of HCV transmission followed by injection drug users with predominance of genotype 3a in both the groups (1 and 2) (Table 3.2.3 and 3.2.4). Pakistan is a country still facing the problems related to transfusable-screened blood for the patients. Reasons might be the poorly equipped resources, weak infrastructure, untrained staff, increasing power breakdowns and ineffective screening of blood donors (Akhtar et al., 2004; Akhtar and Moatter 2004 and Aslam and Syed

2005). Earlier, Masood et al. (2005) reported almost same results from other areas of Pakistan where the blood transfusions were the apparently major cause of disease spread. The overall observed modes of HCV transmission in Pakistan include multiple use of needles/syringes (61.45%), major/minor surgery/dental procedures (10.62%), blood transfusion and blood products (4.26%), sharing razors during shaving or circumcision by barbers (3.90%), and in less than 1% due to needle stick, from infected mother to baby and sexual transmission (Idrees et al., 2008). Moreover, the present data is in conformity with another study conducted in Pakistan which had reported increasing trend of injection drug use (Strathdee et al., 2003).

An association between HCV genotypes and the mode of HCV transmission has been reported by some authors in certain areas of the world (Pawlotsky et al., 1995 and Zein et al., 1996). In Europe, blood transfusion is commonly associated with genotype 1 while intravenous illicit drug use is more common in patients with genotype 3 infection (Simmonds, 2004). It can be proposed that there should be some projects started by the government and non-governmental organizationsfor learning fot eh common people about hepatitis C and its transmission in order to reduce the eminent threat about the spread of disease particularly that of the uncommon genotypes which are moderately less responsive to interferon-based therapies. Policies regarding economic rehabilitation and psychological counseling for the war-affected people should help to minimize the practice of injection drug use. In current study, awareness of the distribution and frequency of the HCV genotypes can provide evidence to better realize the HCV transmission and direct the development of prevention strategies.

4.3 EVOLUTIONARY DYNAMICS AND HCV RESPONSE TO ANTIVIRAL THERAPY

4.3.1 Determination of Phylogeny

Phylogenetic methods are essential tools for the study of molecular epidemiology. These are helpful in determining the degree of genetic diversity in the genome of any organism (Kaneko et al., 1999 and Sakai et al., 1999). HCV isolates show four levels of genetic variability: types, subtypes, isolates, and quasispecies. The degree of sequence divergence between subtypes of different genotypes is surprisingly uniform. This leads to a phylogenetic appearance in which each major branch (genotype) divides into multiple new branches (subtypes) at a similar time in

evolutionary history (Mellor et al., 1996 and Simmonds et al., 1996). This divergence might be due to underlying demographic changes in host population, or structural constraints on genome divergence. Although the classification of new HCV sequences should preferably be based on complete genome sequences, tentative clade/subtype assignments can be based on phylogenetic analysis of nucleotide sequences of at least two coding regions (Robertson et al., 1998 and Quarleri et al., 2000).

Complex epidemiological patterns has been shown by HCV genotypes and subtypes with respect to geographical distribution, transmission modes prevalence, and response to treatment. The recognition of factors responsible for this complex epidemiology is difficult, but will absolutely contribute to a better understanding of HCV genetic dynamics required to establish a preventive strategy of disease control (Jimenez-Hernandez et al., 2007). The association and evolutionary relationship of the species can be traced by construction of phylogenetic trees. Species are generally stable, changing little for millions of years. This smooth phase is "punctuated" by a rapid burst of change resulting in a new form of life and is called punctuated equilibrium (Gould and Niles, 1972), connecting the missing links of evolutionary process. Gould (1977) emphasized that biological evolution takes place in terms of sporadic burst of activity separating relatively long periods of quiescence, rather than in gradual manner. Extinctions are indeed episodic at all scales and fitness landscape represents the ability of species to survive as a function of their genetic code (Bak and Sneppen 1993).

Three coding regions of HCV genome, core, E1, and NS5B were identified initially as being useful for identifying genotypes by distance-based methods (Simmonds et al., 1993; Simmonds et al., 1994; Ohba et al., 1995 and Mizokami, et al., 1996). Different regions of HCV genome show varying levels of sequence diversity and variable relative rate of nucleotide substitutions. The ends of the genome 5′- and 3′- UTRs show little variations (Bukh et al., 1992 and Purcell, 1994), these regions also have the slowest rate of substitution, same is the case with initial coding region (core gene). It has been suggested that examination of the HCV 5′ UTR, although less heterogeneous than other regions of the viral genome, correlates well with and is more sensitive than interrogation of other genomic regions of HCV and is adequate for determining the HCV genotype (Chan et al., 1992; Simmonds et al., 1994b; Dixit et al., 1995; Smith et al., 1995 and Germer et al., 1999). However,

several reports have indicated that analysis of the 5′ UTR cannot accurately differentiate HCV subtypes (Andonov and Chaudhary 1995; Bukh et al., 1995; Germer et al., 1999; Simmonds, 1995; Smith et al., 1995 and Zeuzem et al., 1995). In this regard, alternative genomic regions have been proposed for use in HCV typing. NS5B for example, is useful because it is sufficiently variable which may identify both genotypes and subtypes. Sequence and phylogenetic analysis of variable genomic regions such as the NS5B has been recommended for HCV genotyping and subtyping (White et al., 2000; Simmonds et al., 2005; Nopporpanth et al., 2006 and Ross et al., 2008). Nucleotide sequencing followed by phylogenetic analysis of regions like HCV core and NS5B has also been suggested for HCV genotyping (Chen and Weck, 2002). In viral replication and immunopathogenesis HCV core region is supposed to play an active role. The diagnostic implication of HCV core protein in sera has been reported (Kanto et al., 1994 and Ansari et al., 2012)).

In the current study in order to determine the role of genetic diversity of HCV isolates in antiviral response, phylogenetic trees were constructed on the sequences of 5′ UTR, core and NS5B separately in association with the sequences from all over the world for both the treatment groups using the MP algorithm of MEGA 5. The sequences of all the three genomic regions of both the treatment groups were successfully aligned by using online multiple alignment software ClustalW. Aligned sequences were further subjected to construct trees using the software MEGA5. Evolutionary trees of sequenced samples of both treatment groups i.e those who respond and those who remain non-responders, were constructed to find out the relationships among the sequences, novel genotypes, subtypes and variants. Closely related HCV sequences reported from five continents around the globe were retrieved from Los Alamos National Laboratory (LANL) HCV database to find out the evolutionary relationship between the studied strains and the HCV strains circulating in these continents.

Phylogenetic tree constructed using sequences of 5′ UTR, core and NS5B exhibited more diverge pattern of evolution by both the treatment groups (Figure 3.3.1, 3.3.2 and 3.3.3). A general trend in the dendrograms indicates that actively mutated and newly evolved sequences were mainly unresolved. The reason of being unresolved is the high mutation rate of HCV (Zein, 2000 and Khan et al., 2009) as different geographical and epidemiological patterns determine the genotypes and

subtypes of HCV (Smith et al., 1997; Simmonds, 2004 and Carrington et al., 2005). Most of the Asian strains showed similarity with European and American (North and South) strains might be due to some migration events. Some of the present study strains showed homology with African strains and they lie in the same cluster with African strains. A study describes that there is a sizeable community of South Asians like Asian labor migrants settled in European countries. Currently, approximately 2 million South Asians are living in Africa; some came in late nineteen and early twentieth century (Oonk, 2007). Similarly, a study showed that South American countries are populated with European, Asian and Africans (Collier et al., 1994).

A limited migration pattern has been identified among strains of Europe, North America, South America and Africa that have shown high diversity in their respective geographical regions as reported previously that in areas of endemicity a highly divergent pattern was observed among the strains suggesting long infection duration (Pybus et al., 2009). Some strains did not show any branching pattern giving an idea about absence of any change that has occurred in them for years. This might be because of the high negative selection pressure which they are undergoing due to some environmental factors whereas their non-response to therapy might be due to some other change at nucleotide or amino acid level or some host factors involved. Therefore, here it can be proposed that less diversification leads to positive response towards antiviral therapy.

4.3.2 Nucleotide Sequence Diversity of 5′ UTR, Core AND NS5B

Patients in group 1 (with SVR) showed significantly less number of transitions and genetic distances than the non-responders (group 2) (Table 3.3.1). In addition the observed mutation spots (including all the insertions, deletions, transitions and transversions) were less in patients with SVR (group 1) as compared to non-responders (group 2).

Increase in homogenous viral population associated with viral clearance is a result of intense reduction in genetic diversity independent of genotype. Also in accordance with our results, a surprisingly parallel drift of decreasing viral diversity was recognized just before viral clearance in acute resolving hepatitis, whereas an increase in viral diversity was found to correlate with acute hepatitis that progressed to chronicity (Domingo et al., 1988). For any given genome; mutation rate determines the ability of a virus to maintain essential information while surviving with different

environmental changes (Domingo et al., 1985; Domingo and Holland, 1994 and Coffin, 1995).

The targeted genomic regions (5′ UTR, core and NS5B) in the present study also showed more non-synonymous substitutions than synonymous in group 2 (non-responders) as compared to group 1 (with SVR) (Table 3.3.1). The higher rates of non-synonymous mutations are considered to modify the epitopes and help the virus to evade both the treatment and immune system. In the current study sequences of NS5B showed more variability as compared to the sequences of 5′ UTR and core. In addition, nucleotides 30–170 of 5′ UTR are shown to be highly conserved between the two studied groups as compared to the other two genomic regions (Table 3.3.2). It is evident that genomic regions such as the 5′ UTR and the core are highly conserved; the non-structural regions NS2, 3, 5B and the 3′ UTR are relatively variable, while the envelope regions E1 and E2, NS4 and the NS5A genes displayed the maximum sequence diversity (Le et al., 2007). Irrespective of the mechanism, it is surprising that an RNA virus that superficially does not induce latent infection can persist for long time (weeks to months), in both breakthrough and relapsed patients, in the phase of seemingly apparently complete period of viremia suppression. The most acceptable assumption is that, in spite of disappearance of viremia, very low levels of viral replication continue to occur in the liver, making a persistent reservoir for virus revival after relapse from the suppressive effects of IFN (Domingo et al., 1988). in addition The infected extrahepatic tissues might act as a reservoir for HCV, and play a role in both HCV persistence and reactivation of infection (Yan et al., 2000).

Present results also showed the highest variability in NS5B region of the group 2 who were non-responders. NS5B encoded the RNA-dependent RNA polymerase which is error prone and deficient in proofreading and resulted in random introduction of base changes into the viral genome (Bukh et al., 1995). This become more problematic for the infected patient, as quasispecies variation confers significant adaptive potential on HCV and has been concerned in the evasion and control of the host response to infection and in differential sensitivity to IFN therapy. The hostile antiviral host environment may drive the proliferation of HCV "evasion variants" from a pre-existing quasispecies pool or through viral genetic adaptation (Farci et al., 2002).

4.3.3 3D Protein Structure and Function Prediction

Computational studies have appealed the scientist's community to recognize novel drug targets and fortify the drug development process. The field of computational chemistry and its recent applications has provided reliable tools for the better understanding of biological systems of interest (Barreca et al., 1999; Cheng et al., 2002 and Azam et al., 2009a, b, c, 2010 and 2012). To explore the protein structure and function predictions, models and Ramachandran plots for core and NS5B proteins were built using I-TASSER and PROCHECK (Figures 3.3.4, 3.3.5, 3.3.6 and 3.3.7). Based on these predicted protein models, important sites can be explored for the identification of specific sites required for suitable drug designing.

Core protein is reported to be involved directly or indirectly in hepatocarcinogenesis and steatosis hepatitis (Hope et al., 2002 and Lerat et al., 2002). Furthermore, this protein also interacts with various cellular proteins and affects host cell functions like gene transcription, lipid metabolism, apoptosis and various signaling pathways (Tellinghuisen et al., 2002). After translation is completed, during HCV replication, HCV polyprotein gets cleaved into at least ten distinct products. The order in which cleavages occur from N-terminus to C-terminus is - C-E1-E2-p7-NS2-NS3-NS4A-NS4B-NS5A-NS5B (Lin and Rice 1995). These proteins help virus to maintain its structural integrity and protection against its host as well as incorporate virulence and pathogenesis to the virus such as envelop proteins. Interaction of some phosphorylation sites with kinases might be responsible for HCV resistance to antiviral effects of IFN which could be confirmed by analyzing these sites for different HCV genotypes (Afzal et al., 2011). Therefore, with the help of predicted protein models, prediction of cleavage and phosphorylation sites in HCV polyproteins specifically in NS5B is of prime importance as it can be further targeted for designing an appropriate drug against resistant strains.

4.3.4 Frequency of Amino Acids

A remarked difference was observed in percentage of individual amino acids of core as well as NS5B protein between the both groups (1 and 2) (Figure 3.3.8a and b). Many studies have reported that substitutions in HCV core region results in enhanced insulin resistance, steatosis, oxidative stress and HCC (Hu et al., 2009 and Pazienza et al., 2010). The phylogenetic analysis depicts that viral genome undergone various significant changes with time at different rates in which core region is

considered to be more diverse (Bostan et al., 2010). The study of sequence diversity in HCV core region has additional value since this viral protein has been related with several elements potentially involved in HCV pathogenesis and persistence (Ciccaglione et al., 2007 and Waggoner et al., 2007). Furthermore in *in vitro* studies key positions within NS5B have been identified that can intensely disrupt HCV replication (Lohmann et al., 2000 and Leveque and Wang, 2002). A relationship has been revealed between the replicative capacity and number of amino acid substitutions in NS5B (Itakura et al., 2005 and Le Pogam et al., 2008), in addition to HCV treatment outcome (Hamano et al., 2005).

Therefore, higher number of mutations within NS5B and core may have a harmful effect on viral replication both *in vitro* and *in vivo*. In an infected individual the presence of multiple unique NS5B variants allows rapid viral adaptation to antiviral and immunologic selection pressures and the cellular microenvironment (Blackard et al., 2010). Conversely viral diversification may also be neutral evolution, with outgrowth of most fit variants, rather than selection by immune system (Li et al., 2011).

4.3.5 Analysis of Amino Acid Heterogeneity

Difference in number of conserved and variable sites was observed between the two groups. Moreover, amino acid substitution in conserved sites with multiple substitutions at single site was observed in non-responders whereas in SVR patients, single substitution was observed at these positions (Table 3.3.3 and Figure 3.3.9). This particular pattern of substitution may be an important feature which is involved in the non-responsiveness of HCV to antiviral therapy.

Amino acid mutations cause the changes in protein phenotype and thus considered as most deleterious. Though, some of these substitutions are neutral when the protein function does not affected by mutation and are maintained by genetic drift. Both the genomic regions concerned in the present study are important in the stability of HCV genome and its replication. Earlier, it has also been investigated that amino acid substitution in the HCV core region could be a useful predictor of the virological response to peg-IFNα plus RBV combination therapy (Akuta et al., 2007). Moreover, as NS5B is error prone and lacking the proofreading, therefore any change resulted in random introduction of base leads to changes in the viral genome (Bukh et al., 1995) that might affect its response towards therapy. Therefore, the RdRp is an important

target for the development of anti-HCV drugs (Pawlotsky and McHutchison, 2004; Di Marco et al., 2005; Ma et al., 2005 and Pawlotsky, 2006). Hence, estimation of any change in these genomic regions would be helpful in determining the viral response to therapy and for designing further strategies against HCV.

4.3.6 Prediction of N-Linked Phosphorylation Site

More phosphorylation sites were observed in core protein of group 2 (non-responders) as compared to group 1 (with SVR). Moreover, the amino acid sequences of NS5B did not show any considerable difference in the number of predicted phosphorylation sites between the two groups. Representative predictions among the 75 sequences each for both groups and also for both proteins are given in figure 3.3.10 and 3.3.11.

It is well known that the first defense line against viral infection in mammals is IFN system (Grandvaux et al., 2002). Viral particles replicate inside the host cell and infect the surrounding cells on getting released. However, infected cells also inform the neighboring cells about the viral presence by releasing interferon. In response, the neighboring cells produce large amounts of an enzyme known as PKR which is a serine/threonine kinase found in cells in dormant state. PKR is induced by interferon and activated upon autophosphorylation. It plays an important role in cellular antiviral defense as well as in apoptosis, signal transduction and transformation (Clemens, 1997). Ample evidence is available for the important role of PKR in the antiviral effect of interferon (Sen and Ransohoff, 1993; Lee et al, 1996 and Williams, 1999). HCV has modified itself to avoid the antiviral effects of PKR (Mathews et al., 1996 and Guo et al., 2001) and here in the present study it is thought that phosphorylation of core proteins leads to IFN resistance. Since HCV core interacts and binds to with PKR and this interaction might be a general phenomenon, irrespective of HCV strain and genotype (Yan et al., 2007). The current study focused on detecting such potential phosphorylation sites in our local isolates of HCV who do not respond to therapy. It is further proposed that this interaction might be responsible for HCV resistance to antiviral therapy which could be further confirmed by dephosphorylating these sites and analyzing its effects on PKR binding and inhibition.

4.3.7 Determination of N-Linked Glycosylation Sites

More N-linked glycosylation sites were observed in core and NS5B protein sequences in patients who did not respond to the therapy (group 2) in comparison to

those who showed SVR (group 1) (Figure 3.3.12 and 3.3.13). Viruses use glycosylation pathways by attaching with N-linked oligosaccharides. Folding and trafficking of envelop and other surface proteins with the help of host cellular chaperones and folding factors are promoted by N-glycosylation. The complexity of viral glycoproteins is amplified by the addition or deletion of glycosylation sites during the period of viral evolution. Any alteration in glycosylation sites effect dramatically the transmissibility and survival of the virus and can modify conformation and folding and consequently affecting portions of the entire molecule (Meunie et al., 1999; Land and Braakman, 2001 and Slater-Handshy et al., 2004). Variations in glycosylation alter the receptor interaction and make a virus more recognizable by the intrinsic factors of host immune cells and less recognized by antibody, hence affecting viral infectivity and replication.

Many viruses i.e., influenza, HIV, hepatitis and West Nile virus that influence human health perform important functions in pathogenesis and immune evasion using glycosylation (Vigerust and Shepherd, 2007). Therefore here we can assume that resistant behavior of HCV isolates might be due to the increased glycosylation that protect the viral particle to respond to the therapy.

4.3.8 siRNA Prediction for 5′ UTR

In the current study a computational approach was applied to design siRNA from the 75 obtained sequences of 5'UTR of non-responders (Table 3.3.4). High degree of HCV strain variability leads to unavailability of vaccine. Targeting of HCV structural gene using siRNA can that silence efficiently full length HCV particles and can be an effective therapeutic option against HCV infection (Ansar et al., 2011). The genome of HCV is a positive-stranded RNA comprises a single, long open reading frame that encodes structural and non-structural proteins. IRES, located in the untranslated region at 5′ UTR mediate the viral genome translation (Tsukiyama-Kohara et al., 1992). A considerable variation exists among the genome of different HCV strains. Though, the 5′ UTR and the upstream portion of the core region constitute the conserved parts of the genome, with 99.6 % nucleotide identity (Choo et al., 1991 and Okamoto et al., 1991). Therefore 5′ UTR is considered as an ideal target for siRNA since sequence mismatches between the target and siRNA affect the efficiency of RNAi.

It was also observed that same targeted sites can be used against various genotypes presenting the conserved DNA and RNA domain in this region therefore, single siRNA can be used against various genotypes and inhibit HCV RNA synthesis. In addition efficient and safe delivery methods are required to suppress HCV replication in infected cells. Here it can be assumed that computationally designed siRNA can inhibit replication of HCV inside the cells recommends that this approach of RNA-targeting might deliver an actual therapeutic option against HCV infection, especially at the optimal site within the conserved 5'UTR.

However, the currently used approach to design siRNA is just an elementary computational approach. Though by using these theoretical findings the functional siRNA can be synthesized that required animal study (*in vitro* and *in vivo*) before coming in practice which cannot be done in the present study due to lack of facilities at present.

4.4 FUTURE PLANS

Based on the data generated from the present study, following parameters have been identified for future activities;

1. To analyze the host genetics (e.g. IL28b polymorphism)
2. To characterize more genomic regions of HCV isolates
3. Identification of genetic variants of HCV prevailing in host as minor population
4. siRNA would be verified in both *in vitro* and *in vivo* studies.

CONCLUSION

Hepatitis C virus (HCV) is the most destructive one among the other blood borne pathogens and causes a major health issue worldwide. Eradication of virus is the main aim of treatment against HCV defined by sustained virological response (SVR). In serum a quick reduction of HCV RNA levels was observed by IFN treatment while long term responses became obvious with persistent loss of HCV RNA in serum. However, in some cases antiviral treatment is unable to clean up the viral RNA in serum. Therefore, it is of great importance to get insight the mechanisms involved in non-responsiveness and further to recognize the factors that can predict the likelihood of patient's response towards treatment. Various viral and host factors are involved in non-responsiveness besides the mechanisms stimulated by HCV proteins at molecular level to reduce the IFN signaling pathway. Present study was directed to find out the host and viral elements involved in viral resistance to therapy.

Among the selected 500 ELISA positive patients, 451 started therapy and were grouped on the basis of their treatment response i.e., group 1 include 376 patients, who were responding positively to therapy while group 2 define the non- responders and constitute the 75 patients. To evaluate the host factors the seroepidemiology was checked for which both the treatment groups were compared with positive and negative control groups. Serum markers included liver profile, lipid profile and some selected blood parameters were evaluated throughout the duration of therapy till the end. Normalization of serum markers was observed in group 1 while no obvious change was observed in non-responders. Furthermore, to determine the viral factors, genetic analysis of the viral strains was performed by targeting three genomic regions 5'UTR, core and NS5B. As the group 2 comprised the 75 non-responders, also 75 patients from 376 patients of group 1 were randomly picked to perform the different analysis of the obtained sequences. Nucleotide sequences were translated into amino acid sequences using online tool TRANSLATE. Sequence variability of the HCV isolates was determined by dragging the dendrograms using MEGA 5 with reference sequences retrieved from the database.

Prevalence of genotype 3 was observed in group 1 while group 2 exhibited some rare genotypes i e. 4 and 5 while genotype 6q and 6v were reported first time from Pakistan. High genetic diversity, increased mutation rate at both nucleotide and amino acid level and high non-synonymous substitution rate in nucleotide sequences was observed in non-responders as compared to responders using BioEdit version 7. 3D protein models were predicted using online software i-TASSER and were further evaluated using another software PROCHECK. Ramachandran plots obtained from PROCHECK with more than 90 % residues in the favored regions were referred as good for further analysis. Amino acid frequency was determined using MEGA 5 presented the difference in percentage composition of individual amino acids of both the proteins between the two groups. Whereas amino acid heterogeneity analysis using DNAman revealed more variability and different pattern of amino acid substitution in non-responders as compared to patients with SVR. More glycosylation sites were observed using SignalP-NN and also more phosphorylation sites using NetPhos server 2.0 were calculated in non-responders as compared to responders. In addition to explore the determinants of viral response, a computational effort was put to design the siRNA using software siDirect supposed to block the HCV RNA synthesis. Most of the predicted siRNA exhibit the same domain and can be used against more than one genotype. Hence, on the basis of overall results it can be concluded that besides host immune response, viral genetic heterogeneity plays very important role in HCV response to therapy. There should be some therapeutic regimes that can overcome the genetic diversity of the viral isolates, to control the phosphorylation and glycosylation of the viral proteins. Moreover, the siRNA should become common practice against viral endemic in future.

Major achievements from the study so far includes a research paper entitled "Hepatitis C Viral Heterogeneity Based on Core Gene and an Attempt to Design Small Interfering RNA Against Strains Resistant to Interferon in Rawalpindi, Pakistan" published in "Hepatitis Monthly" and another research article "Global diversity of HCV: *In-silico* analysis based on core region" has been published in Romanian

Biotechnological Letters. One review article is under peer review by respective journal. Two abstracts from this study were accepted for poster presentations one in International Conference on Emerging Infectious Diseases held in Hyatt Regency Atlanta Georgia held on March 11th to 14th 2012 while second one in "22nd European Congress of Clinical Microbiology and Infectious Diseases" held on March 31st to April 4th 2012 in United Kingdom. Moreover an abstract was also accepted for oral presentation in "1st National Symposium on New Horizons of Microbiology": Steps of Microbes on Leader of Life" held on November 7th to 8th 2012 in Federal Urdu University Karachi Pakistan.

REFERENCES

Abdulkarim AS, Zein NN, Germer JJ, Kolbert CP, Kabbani L, Krajnik KL, Hola A, Agha MN, Tourogman M and Persing DH (1998). Hepatitis C virus genotypes and hepatitis G virus in hemodialysis patients from SySria: identification of two novel hepatitis C virus subtypes. Am J Trop Med Hyg. 59: 571-576.

Afdhal N, McHutchison J, Brown R, Jacobson I, Manns M, Poordad F, Weksler B and Esteban R (2008). Thrombocytopenia associated with chronic liver disease. J Hepatol. 48(6): 1000-1007.

Afridi S, Naeem M, Hussain A, Kakar N, Babar ME and Ahmad J (2009). Prevalence of hepatitis C virus (HCV) genotypes in Balochistan. Mol Biol Rep. 36(6):1511-4.

Afzal S, Idrees M, Ali M, Ilyas M, Hussain A, Akram M, Sadia A, Butt SS, Rehamn I, Ali L and Shahid M (2011). Envelope 2 protein phosphorylation sites S75 & 277 of hepatitis C virus genotype 1a and interferon resistance: a sequence alignment approach. Virol J. 8: 71.

Ahmad S, Salati SAA, Mattar EH, Al-Sabban AMH and Hamad AM (2010). Epidemiology of Hepatitis C Virus (HCV) Infection. Phys Acad. 4(8): 82-87.

Ahmad W, Ijaz B, Javed FT, Kausar H, Sarwar MT, Gull S, Asad S, Imran S and Hassan S (2011). HCV genotype-specific correlation with serum markers: Higher predictability for genotype 4a. Virol J. 8:293.

Akbar H, Idrees M, Manzoor S, Rehman I, Butt S, Yousaf MZ, Rafique S, Awan Z, Khubaib B, Akram M and Aftab M (2009). Hepatitis C virus infection: A review of the current and future aspects and concerns in Pakistan. J Gen Mol Virol. 1(2): 012-018.

Akhtar S and Moatter T (2004). Intra-household clustering of hepatitis C virus infection in Karachi, Pakistan. Transactions of the Royal Society of Tropical Medicine and Hygiene. 98(9): 535-539.

Akhtar S, Younus M, Adil S, Jafri SH and Hassan F (2004). Hepatitis C virus infection in asymptomatic male volunteer blood donors in Karachi, Pakistan. J Vir Hepat. 11(6):527-535.

Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Kobayashi M, Arase Y, Ikeda K and Kumada H (2007). Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b: amino acid substitutions in the core region and low-density lipoprotein cholesterol levels. J Hepatol. 46: 403–410.

Al RH, Xie YP, Wang YH and Hagedorn CH (1998). Expression of recombinant hepatitis C virus non-structural protein 5B in Escherichia coli. Vir Res. 53: 141-149.

Albadalejo J, Alonso R, Antinozzi R, Bogard M, Bourgault AM, Colucci G, Fenner T, Petersen, Sala HE, Vincelette J and Young C (1998). Multicenter evaluation of the COBAS AMPLICOR HCV assay, an integrated PCR system for rapid detection of hepatitis C virus RNA in the diagnostic laboratory. J Clin Microbiol. 36: 862-865.

Alberti A, Benvegnu L, Boccato S and Ferrari G (2004). Natural history of initially mild chronic hepatitis C. Dig Liver Dis. 36(10): 646-654.

Al-Khurri LE, Al-Khafaji KR, Al-Salihi SA, Alwaysi SAA and Al-Akayshi RJ (2009). Serum HCV-RNA levels in patients with chronic hepatitis C: Correlation with histological features. Arab J Gastroenterol. 10: 10-13.

Al-Moslih MI and Al-Huraibi MA (2001). Prevalence of Hepatitis C virus among patients with liver disease in the republic of Yemem.East. Mediter Health J. 7(4-5):771-778.

Alter MJ (2007). Epidemiology of heaptitis C virus infection. W J Gastroenterol. 13(17): 2436-2441.

Alter MJ, Kruszon-Moran D, Nainan OV, McQuillan GM, Gao F, Moyer LA, Kaslow RA and Margolis HS (1999). The prevalence of hepatitis C virus infection in the United States, 1988 through 1994. N Engl J Med. 341: 556-562.

Andonov A and Chaudhary RK (1995). Subtyping of hepatitis C virus isolates by a line probe assay using hybridization. J Clin Microbiol. 33: 254–256.

Ank N, Iversen MB, Bartholdy C, Staeheli P, Hartmann R, Jensen UB, Dagnaes-Hansen F, Thomsen AR, Chen Z, Haugen H, Klucher K and Paludan SR (2008). An important role for type III interferon (IFN-lambda/IL-28) in TLR-induced antiviral activity. J Immunol. 180(4): 2474–2485.

Ank N, West H, Bartholdy C, Eriksson K, Thomsen AR and Paludan SR (2006). Lambda interferon (IFN-lambda), a type III IFN, is induced by viruses and IFNs and displays potent antiviral activity against select virus infections in vivo. J Virol. 80:4501-4509.

Ansar M, Ashfaq UA, Shahid I, Sarwar MT, Javed T, Rehman S, Hassan S and Riazuddin S (2011). Inhibition of full length Hepatitis C Virus particles of 1a genotype through small interference RNA. J Virol. 8: 203.

Ansari MA, Lingaiah R and Irshad M (2012) HCV-Core Region: Its Significance in HCV-Genotyping and Type Dependent Genomic Expression. Maced J Med Sci. 15: 5(1):30-39

Aral SO, Fenton KA and Holmes KK (2007). Sexually transmitted diseases in the USA: temporal trends. Sex Transm Infect. 83: 257-266.

Araujo FMG, Sonoda IV, Rodrigues NB, Teixeira R, Redondo RAF and Oliveria GC (2008). Genetic variability in the 5' UTR and NS5A regions of hepatitis C virus RNA isolated from non-responding and responding patients with chronic HCV genotype 1 infection. Mem Inst Oswaldo Cruz. 103(6): 611-614.

Aref S, Sleem T, El Menshawy N, Ebrahiem L, Abdella D, Fouda M, Samara NA, Menessy A, Abdel-Ghaffar H, Bassam A and Abdel Wahaab M (2009). Anti-platelet antibodies contribute to thrombocytopenia associated with chronic hepatitis C virus infection. Hematol. 14: 277–281.

Asif N, Zafar T, Hassan K and Naseem L (2009). Seroprevalence Anti HCV Antibodies, HCV- RNA and its Genotypes among Patients of Hemophilia, at Hemophilia Treatment Centre Pakistan Institute of Medical Sciences, Islamabad. Int J Pathol. 7(2): 84-87.

Aslam F and Syed JA (2005). Seeking a safer blood supply. Lancet. 365(9469): 1464-1464.

Asselah T, Estrabaud E, Bieche I, Lapalus M, De Muynck S, Vidaud M, Saadoun D, Soumelis V and Marcellin P (2010). Hepatitis C: viral and host factors associated with non-response to pegylated interferon plus ribavirin. Liver Int. 30 (9): 1259-69.

Attaullah S, Khan S and Ali I (2011). Hepatitis C virus genotypes in Pakistan: a systemic review. Virol J. 8: 433.

Azam SS, Hofer TS, Bhattacharjee A, Lim LHV, Pribil AB, Randolf BR and Rode BM (2009a). Beryllium (II): the Strongest Structure- Forming Ion in Water? A QMCF MD simulation study. J Phys Chem B 113(27):9289–9295

Azam SS, Hofer TS, Randolf BR and Rode BM (2009b). Hydration of sodium(I) and potassium(I) revisited: a comparative QM/MM and QMCF MD simulation study of weakly hydrated ions. J Phys Chem A. 113:1827–1834.

Azam SS, Hofer TS, Randolf BR and Rode BM (2009c). Germinum(II) in water: an unsual hydration structure results of a QMCF MD simulation. Chem Phys Lett. 470:85–89.

Azam SS, Lim LHV, Hofer TS, Randolf BR and Rode BM (2010). Hydrated germanium (II): irregular structural and dynamical properties revealed by a quantum mechanical charge field molecular dynamics study. J Comput Chem. 31(2):278–285

Azam SS, Uddin R, Adnan AS and Haq Z (2012). Molecular docking studies of potent inhibitors of tyrosinase and a-glucosidase. Med Chem Res. 21:1677–1683

Azzari C, Resti M, Moriondo M, Ferrari R, Lionetti P and Vierucci A (2000). Vertical transmission of HCV is related to maternal peripheral blood mononuclear cell infection. Blood. 96:2045-2048.

Bak P and Sneppen K (1993). Punctuated equilibrium and criticality in a simple model of evolution. Phy Rev Lett. 71 (24): 4083-4086.

Ballem PJ, Belzberg A, Devine DV, Lyster D, Spruston B, Chambers H, Doubroff P and Mikulash K (1992). Kinetic studies of the Mechanism of thrombocytopenia in patients with human immunodeficiency virus infection. N Engl J Med. 327: 1779–1784.

Balvey T (2009). Structural and functional diversity of viral IRESes. Biochim Biophys Acta. 1789: 542-557.

Barreca ML, Carotti A, Carrieri A, Chimirri A, Monforte AM, Calace MP and Rao A (1999). Comparative molecular field analysis (CoMFA) and docking studies of non-nucleoside HIV-1 RT inhibitors (NNIs). Bioorg Med Chem 7(11):2283–2292.

Bedimo R, Ghurani R, Nsuami M, Turner D, Kvanli MB, Brown G and Margolis D (2006). Lipid abnormalities in HIV/hepatitis C virus-coinfected patients. HIV Med. 7(8): 530-6.

Beld M, Sentjens R, Rebers S, Weel J, Wertheim-van D, Sol C and Boom R (2000). Detection and quantitation of hepatitis C virus RNA in Feces of chronically infected individuals. J Clin Microbiol. 38(9): 3442-3444.

Bell H, Hellum K and Harthug S (1997). Genotype, viral load and age as independent predictors of treatment outcome of interferon-a 2a treatment in patients with chronic hepatitis C. Scand J Infect Dis. 29: 17–22.

Bellentani S and Tiribelli C (2001). The spectrum of liver disease in the general population: lesson from the Dionysus study. J Hepatol. 35(4): 531-537.

Bissell DM (1999). Sex and hepatic fibrosis. Hepatol. 29988–989.989.

Blackard JT, Gang Ma, Limketkai BN, Welge JA, Dryer PD, Martin CM , Hiasa Y, Taylor LE, Mayer KH, Jamieson DJ and Sherman KE (2010). Variability of the Polymerase Gene (NS5B) in Hepatitis C Virus-Infected Women. J Clin Microbiol. 48(1):4256-4259.

Bonfanti P, Landonio S, Ricci E, Martinelli C, Fortuna P, Faggion I, Quirino T and CISAI study group (2001). Risk factors for hepatotoxicity in patients treated with highly active antiretroviral therapy. J AIDS. 27: 316-318.

Bonkovsky HL and Mehta S (2001). Hepatitis C: a review and update. J Am Acad Dermatol. 44(2): 159-182.

Bordin G, Ballaré M, Zigrossi P, Bertoncelli MC, Paccagnino L, Baroli A, Brambilla M, Monteverde A and Inglese E (1995). A laboratory and thrombokinetic study of HCV-associated thrombocytopenia: a direct role of HCV in bone marrow exhaustion? Clin Exp Rheumatol. 13: S39-S43.

Bostan N (2010). Molecular epidemiology and genetic characterization of Hepatitis C Virus in patients from suburban Rawalpindi, Pakistan. DRSM library, Plant Department, Biological Sciences, QAU.

Bostan N and Mahmood T (2010). An Overview about Hepatitis C: A Devastating Virus. Crit Rev Microbiol. 36(2): 91-133.

Bostan N, Mustafa MM, Safdar W, Javed Q and Mahmood T (2010). Phylogenetics of HCV: Recent advances. A J Biotechnol. 9(36):5792-5799.

Bouchardeau F, Cantaloube JF, Chevaliez S, Portal C, Razer A, Lefrère JJ, Pawlotsky JM, De Micco P and Laperche S (2007). Improvement of hepatitis C virus (HCV) genotype determination with the new version of the INNO-LiPA HCV assay. J Clin Microbiol. 45(4): 1140-5.

Boulestin A, Kamar N, Sandres-Sauné K, Legrand-Abravanel F, Alric L, Vinel JP, Rostaing L and Izopet J (2006). Twenty-four hour kinetics of hepatitis C virus and antiviral effect of alpha-interferon. J Med Virol. 78: 365-71.

Brinton MA and Dispoto JH (1988). Sequence and secondary structure analysis of the 5'-terminal region of flavivirus genome RNA. Virol. 162: 290-299.

Budowle B, Johnson DM, Fraser CM, Leighton TJ, Murch RS and Chakraborty R (2005). Genetic analysis and attribution of microbial forensics evidence. Criti Rev Microbiol. 31(4): 233-254.

Bukh J, Miller RH and Purcell RH (1995). Genetic heterogeneity of hepatitis C virus: quasispecies and genotypes. Semin Liver Dis. 15(1): 41-63.

Bukh J, Purcell RH and Miller RH (1992). Importance of primer selection for the detection of hepatitis C virus RNA with the polymerase chain reaction assay. Proc Natl Acad Sci USA. 89: 87-91.

Candotti D, Temple J, Sarkodie F and Allain JP (2003). Frequent Recovery and Broad Genotype 2 Diversity Characterize Hepatitis C Virus Infection in Ghana, West Africa. J Virol. 77(14): 7914-7923.

Carlsson T, Reichard O, Norkrans G, Bläckberg J, Sangfelt P, Wallmark E and Weiland O (2005). Hepatitis C virus RNA kinetics during the initial 12 weeks treatment with pegylated interferon-alpha 2a and ribavirin according to virological response. J Viral Hepat. 12(5): 473-80.

Carrington CV, Foster JE, Pybus OG, Bennett SN and Holmes EC (2005). Invasion and maintenance of dengue virus type 2 and type 4 in the Americas. J Virol. 79: 14680-14687.

Chamberlain R. Adams W, Saeed N, Simmonds AA and Elliott P (1997). Complete nucleotide sequence of a type 4 hepatitis C virus variant, the predominant genotype in the Middle East. J Gen Virol. 78: 1341–1347.

Chan SW, McOmish F, Holmes EC, Dow B, Peutherer JF, Follett E, Yap PL and Simmonds P (1992). Analysis of a new hepatitis C virus type and its phylogenetic relationship to existing variants. J Gen Virol. 73:1131–1141.

Chang KM, Rehermann B, McHutchison JG, Pasquinelli C, Southwood S, Sette A and Chisari FV (1997). Immunological significance of cytotoxic T lymphocyte epitope variants in patients chronically infected by the hepatitis C virus. J Clin Invest. 100: 2376–2385.

Chen SL and Morgan TR (2006). Natural history of the hepatitis C virus (HCV) infection. Int J Med Sci. 3(2): 47-52.

Chen Z and Weck K (2002). Hepatitis C virus genotyping: Interrogation of the 5'untranslated region cannot accurately distinguish genotypes 1a and 1b. J Clin Microbiol. 40: 3127-3134.

Cheng F, Shen J, Luo X, Zhu W, Gu J, Ji R, Jiang H and Chen K (2002) Molecular docking and 3-D-QSAR studies on the possible antimalarial mechanism of artemisinin analogues. Bioorg Med Chem. 10(9):2883–2891.

Chiang JY (2005). Nuclear receptor regulation of lipid metabolism: potential therapeutics for dyslipidemia, diabetes, and chronic heart and liver diseases. Curr Opin Investig Drugs. 6(10):994-1001.

Choo QL, Richman K H, Han J H, Berger K, Lee C, Dong C, Gallegos C, Coit D, Medina-Selby R and Barr PJ (1991). Genetic organization and diversity of the hepatitis C virus. Proc Natl Acad Sci USA. 88: 2451-455.

Chung RT and Kaplan LM (1999). Heterogeneous nuclear ribonucleoprotein I (hnRNP-I/PTB) selectively binds the conserved 3' terminus of hepatitis C viral RNA. Bioch Biophys Res Commun. 254: 351-362.

Ciccaglione AR, Stellacci E, Marcantonio C, Muto V, Equestre M, Marsili G, Rapicetta M and Battistini A (2007). Repression of Interferon Regulatory Factor 1 by Hepatitis C Virus Core Protein Results in Inhibition of Antiviral and Immunomodulatory Genes. J Virol. 81(1): 202–214.

Clemens MJ (1997). PKR--a protein kinase regulated by double-stranded RNA. Int J Biochem Cell Biol. 29: 945-949.

Coffin JM (1995). HIV population dynamics in vivo: implications for genetic variation, pathogenesis, and therapy. Science. 267(5197): 483-489.

Collier, Simon, Skidmore TE and Blakemore H (1994). The Cambridge Encyclopedia of Latin America and the Caribbean (2nd ed). Cambridge, Cambridge University Press.

Curran R, Jameson CL, Craggs JK, Grabowska AM, Thomson BJ, Robins A, Irving WL and Ball JK (2002). Evolutionary trends of the first hypervariable region

of the hepatitis C virus E2 protein in individuals with differing liver disease severity. J Gen Virol. 83(1): 11–23.

Dagan S, Eren R, Graham N, Nussbaum O, Terkieltaub D, Terrault N, Tur-Kaspa R, Lurie Y, Safadi R, Akerman Z and Galun E (2003). Clinical evaluation of a human monoclonal antibody against the envelope protein (E2) of HCV for prevention of HCV infection. Hepatol. 38(4): 302A-302A

Dalgard O, Bjoro K and Hellum KB (2004). Treatment with pegylated interferon and ribavarin in HCV infection with genotype 2 or 3 for 14 weeks: a pilot study. Hepatol. 40: 1260-5.

Daniel CEJKA, Losert D and Wacheck V (2006). Short interfering RNA (siRNA): tool or therapeutic? Clin Sci.110:47–58

Davidson F, Simmonds P, Ferguson JC, Jarvis LM, Dow BC, FoHett EA, Seed CRG, Krusius T, Lin C, Medgyesi GA, Kiyokawa H, Olim G, Duraisamy G, Cuypers TT, Saeed AA, Teo D, Conradie J, Kew MC, Lin M, Nuchaprayoon C, Ndimbie OK and Yap PL (1995). Survey of major genotypes and subtypes of hepatitis C virus using RFLP of sequences amplified from the 5' non-coding region. J Gen Virol. 76: 1197-1204.

Davis GL, Esteban-Mur R, Rustgi V, Hoefs J, Gordon SC, Trepo C, Shiffman ML, Zeuzem S, Craxi A, Ling MH and Albrecht J (1989). Treatment of chronic hepatitis C with recombinant interferon alfa: A multicenter randomized, controlled trial. N Engl J Med. 321(22): 1501-1506.

Davis GL, Lau JY, Urdea MS, Neuwald PD, Wilber JC, Lindsay K, Perrillo RP and Albrecht J (1994). Quantitative detection of hepatitis C virus (HCV) RNA by a solid-phase signal amplicon method: definition of optimal conditions for specimen collection and clinical application in interferon-treated patients. Hepatol.19: 1337-1341.

de Almeida AJ, Campos-de-Magalhaes M, de Melo Marcal OP, Brandao-Mello CE, Okawa MY, de Oliveira RV, do Espirito-Santo MP, Yoshida CF and Lampe E (2004). Hepatitis C virus-associated thrombocytopenia: a controlled prospective, virological study. Ann Hematol. 83: 434-440.

de Moliner L, Pontisso P, de Salvo GL, Cavalletto L, Chemello L and Alberti A (1998). Serum and liver HCV RNA levels in patients with chronic hepatitis C: correlation with clinical and histological features. Gut. 42: 856-860.

Dev AT, McCaw R, Sundararajan V, Bowden S and Sievert W (2002). Southeast Asian patients with chronic hepatitis C: the impact of novel genotypes and race on treatment outcome. Hepatol. 36: 1259–1265.

Di Bisceglie AM, Conjeevaram HS, Fried MW, Sallie R, Park Y, Yurdaydin C, Swain M, Kleiner DE, Mahaney K and Hoofnagle JH (1995). Ribavirin as therapy for chronic hepatitis C. A randomized, double-blind, placebo-controlled trial. Ann Intern Med. 123: 897-903.

Di Marco S, Volpari C, Tomei L, Altamura S, Harper S, Narjes F, Koch U, Rowley M, De Francesco R, Migliaccio G and Carfi A (2005). Interdomain communication in hepatitis C virus polymerase abolished by small molecule inhibitors bound to a novel allosteric site. J Biol Chem. 280:29765–29770.

Diaz O, Delers F, Maynard M, Dmignot S, Zoulim F and Chambaz J (2006). Preferential association of hepatitis C virus with apolipoprotien B48-containing lipoproteins. J GenVirol. 87: 2983-2991.

Dixit V, Quan S, Martin P, Larson D, Brezina M, Dinello R, Sra K, Lau JYN, Chien D and Kolberg J (1995). Evaluation of a novel serotyping system for hepatitis C virus: strong correlation with standard genotyping methodologies. J Clin Microbiol. 33: 2978–2983.

Dolganiuc A, Chang S, Kodys K, Mandrekar P, Bakis G, Cormier M and Szabo G (2006). Hepatitis C virus (HCV) core protein-induced, monocyte-mediated

mechanisms of reduced IFN-alpha and plasmacytoid dendritic cell loss in chronic HCV infection. J Immunol. 177(10): 6758–6768.

Domingo E and Holland JJ (1994). Mutation rates and rapid evolution of RNA viruses. In: Raven (ed.). Evol Biol Vir. New York, 161-184.

Domingo E, Holland JJ and Ahlquist P (1988). RNA Genetics. Boca Raton, FL: CRC Press.

Domingo E, Martinez-Salas E, Sobrino F, de la Torre JC, Portela A, Ortin J, Lopez-Galindez C, Perez-Brena P, Villanueva N, Najera R, VandePol S, Steinhauer D, DePolo N and Holland J (1985). The quasispecies (extremely heterogeneous) nature of viral RNA genome populations: biological relevance--a review. Gene. 40 (1): 1-8.

Doyle SE, Schreckhise H, Khuu-Duong K, Henderson K, Rosler R, Storey H, Yao L, Liu H, Barahmand-pour F, Sivakumar P, Chan C, Birks C, Foster D, Clegg CH, Wietzke-Braun P, Mihm S and Klucher KM (2006). Interleukin-29 uses a type 1 interferon-like program to promote antiviral responses in human hepatocytes. Hepatol. 44(4): 896–906.

Dubuisson J, Helle F and Cocquerel L (2008). Early life cycle of the hepatitis life cycle. Cell Microbiol. 10(4): 821-827.

Dufour DR, Lott JA, Nolte FS, Gretch DR, Koff RS and Seeff LB (2000). Diagnosis and monitoring of hepatic injury. I. Performance characteristics of laboratory tests. Clin Chem. 46: 2027-2049.

Durante ME, Forton DM, Ruggiero G and Karayiannis P (2003). Hepatitis C virus E2 and NS5A region variability during sequential treatment with two interferon-alpha preparations. J Med Virol. 70(1): 62–73

Duverlie G, Khorsi H, Castelain S, Jaillon O, Izopet J, Lunel F, Eb F, Penin F and Wychowski C (1998). Sequence analysis of the NS5A protein of European

hepatitis C virus 1b isolates and relation to interferon sensitivity. J Gen Virol. 79: 1373–1381.

Eissa LA, Gad LS, Rabie AM and El-Gayar AM (2008). Thrombopoietin level in patients with chronic liver diseases. Ann Hepatol. 7: 235–244.

el-Zayadi A, Simmonds P, Dabbous H, Prescott L, Selim O and Ahdy A (1996). Response to interferon-alpha of Egyptian patients infected with hepatitis C virus genotype 4. J Viral Hepat. 3: 261-264.

Espanol I, GallegoA, Rabella N, Lerma E, Hernandez A and Pujol-Moix N (2000). Thrombocytopenia associated with liver cirrhosis and hepatitis C viral infection: role of thrombopoietin. Hepato Gastroenterol. 47: 1404-1406.

European Association for the study of the Liver (1999). EASL international consensus conference on hepatitis C. Consensus statement. J Hepatol. 30: 956-961.

Fabris C, Federico E and Soardo G (1997). Blood lipids of patients with chronic hepatitis: Differences related to viral etiology. Clin Chem Acta. 261: 159-161.

Farci P, Alter HJ, Govindarajan S, Wong DC, Engle R, Lesniewski RR, Mushahwar IK, Desai SM, Miller RH, Ogata N and Purcell RH (1992). Lack of protective immunity against reinfection with hepatitis C virus. Science. 258(5079): 135-140.

Farci P, Shimoda A, Coiana A, Diaz G, Peddis G, Melpoder JC, Strazzera A, Chien DY, Munoz SJ, Balestrieri A, Purcell RH and Alter HJ (2000). The outcome of acute hepatitis C predicted by the evolution of the viral quasispecies. Science. 288: 339-344.

Farci P, Strazzera R, Alter HJ, Farci S, Degioannis D, Coiana A, Peddis G, Usai F, Serra G, Chessa L, Diaz G, Balestrieri A and Purcell RH (2002). Early changes in hepatitis C viral quasispecies during interferon therapy predict the therapeutic outcome. Proc Natl Acad Sci USA. 99(5): 3081-3086.

Flint M and McKeating JA (1999). The C-terminal region of the hepatitis C virus E1 glycoprotein confers localization within the endoplasmic reticulum. J Gen Virol. 80: 1943-1947.

Flisiak R, Feinman SV, Jablkowski M, Horban A, Kryczka W, Pawlowska M, Heathcote JE, Mazzella G, Vandelli C, Nicolas-Métral V, Grosgurin P, Liz JS, Scalfaro P, Porchet H and Crabbé R (2009). The cyclophilin inhibitor Debio 025 combined with PEG IFNalpha2a signi?cantly reduces viral load in treatment-naive hepatitis C patients. Hepatol. 49: 1460–8.

Forns X, Ampurdanès S, Llovet JM, Aponte J, Quintó L, Martínez-Bauer E, Bruguera M, Sánchez-Tapias JM and Rodés J (2004). Identification of chronic hepatitis C patients without hepatic fibrosis by a simple predictive model. Hepatol. 39: 862-3.

Foster GR, Hézode C, Bronowicki JP, Carosi G, Weiland O, Verlinden L, van Heeswijk R, van Baelen B, Picchio G and Beumont M (2011). Telaprevir alone or with peginterferon and ribavirin reduces HCV RNA in patients with chronic genotype 2 but not genotype 3 infections. Gastroenterol. 141(3): 881-9 e.

Franciscus A (2006). HCV Treatment: predictors of Treatment Response. Hepatitis C Support Project. www.hcvadvocate.org.

Freeman AJ, Marinos G, Ffrench RA and Lloyd AR (2001). Immunopathogenesis of hepatitis C virus infection. Immunol Cell Biol.79, 515–536.

Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL Jr, Haussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Lin A, Hoffman J and Yu J (2002). Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med. 347: 975-982.

Gale M Jr and Foy EM (2005). Evasion of intracellular host defence by hepatitis C virus. Nature. 436: 939-45.

Gale M Jr, Kwieciszewski B, Dossett M, Nakao H and Katze MG (1999). Antiapoptotic and oncogenic potentials of hepatitis C virus are linked to interferon resistance by viral repression of the PKR protein kinase. J Virol. 73: 6506-6516.

Gale MJ, Korth MJ, Tang NM, Tan SL, Hopkins DA, Dever TE, Polyak SJ, Gretch DR and Katze MG (1997). Evidence that hepatitis C virus resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein. Virol. 230(2): 217 227.

Gao B, Hong F and Radaeva S (2004). Host factors and failure of interferon-alpha treatment in hepatitis C virus. Hepatol. 39(4): 880-90.

Gastaminza P, Cheng G, Wieland S, Zhong J, Liao W and Chisari FV (2007). Cellular determinants of hepatitis C virus assembly, maturation, degradation and secretion. J Virol. 82(5): 2120-2129.

Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, Heinzen EL, Qiu P, Bertelsen AH, Muir AJ, Sulkowski M, McHutchison JG and Goldstein DB (2009). Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 461:399-401

Genovese D, Dettori S, Argentini C, Villano U, Chionne P, Angelico M and Rapicetta M (2005). Molecular Epidemiology of Hepatitis C Virus Genotype 4 Isolates in Egypt and Analysis of the Variability of Envelope Proteins E1 and E2 in Patients with Chronic Hepatitis. J Clin Microbiol. 43(4): 1902-1909.

Germer JJ and Zein NN (2001). Advances in the molecular diagnosis of hepatitis C and their clinical implications. Mayo Clinic Proceedings. 76(9):911-920.

Germer JJ, Rys PN, Thorvilson JN and Persing DH (1999). Determination of hepatitis C virus genotype by direct sequence analysis of products generated with the Amplicor HCV test. J Clin Microbiol. 37:2625– 2630.

Germer JJ, Rys PN, Thorvilson JN and Persing DH (1999). Determination of hepatitis C virus genotype by direct sequence analysis of products generated with the Amplicor HCV test. J Clin Microbiol. 37:2625– 2630.

Giannini C and Brechot C (2003). Hepatitis C virus biology. Nature. 10: S27-S38.

Giannini E, Botta F, Borro P, Malfatti F, Fumagalli A, Testa E, Podestà E, Chiarbonello B, Polegato S, Mamone M and Testa R (2003). Relationship between thrombopoietin serum levels and liver function in patients with chronic liver disease related to hepatitis C virus infection. Am J Gastroenterol. 98: 2516 -2520.

Gould SJ (1977). Evolution's erratic pace. Nat Histor. 86(5): 12-16.

Gould SJ and Niles E (1972). Punctuated equilibria: an alternative to phyletic gradualism. In T.J.M. Schopf, ed., Models in Paleobiology. San Francisco: Freeman, Cooper and Company. 82-115.

Grandvaux N, tenOever BR, ServantMJ and Hiscott J (2002). The interferon antiviral response: from viral invasion to evasion. Curr Opin Infect Dis. 15: 259-267.

Guo JT, Bichko VV and Seeger C (2001). Effect of alpha interferon on the hepatitis C virus replicon. J Virol. 75: 8516-8523.

Guy R, Gold J, Calleja JMG, Kim AA, Parekh B, Busch M, Rehle T, Hargrove J, Remis RS and Kaldor JM (2009). Accuracy of serological assays for detection of recent infection with HIV and estimation of population incidence: a systematic review. Lanc Infect Dis. 9(12): 74-759.

Hamano K, Sakamoto N, Enomoto N, Izumi N, Asahina Y, Kurosaki M, Ueda E, Tanabe Y, Maekawa S, Itakura J, Watanabe H , Kakinuma S and Watanabe M (2005). Mutations in the NS5B region of the hepatitis C virus genome correlate with clinical outcomes of interferon-alpha plus ribavirin combination therapy. J Gastroenterol Hepatol. 20(9): 1401-1409.

Hayashi J, Kawakami Y and Nabeshima A (1998). Comparison of HCV RNA levels by branched DNA probe assay and by competitive polymerase chain reaction to predict effectiveness of interferon treatment for patients with chronic hepatitis C virus. Dig Dis Sci. 43: 384-391.

Henderson DK (2003). Managing Occupational Risks for Hepatitis C Transmission in the Health Care Setting. Clin Microbiol Rev. 16(3): 546-568.

Herrine SK (2002). Approach to the patient with chronic hepatitis C virus infection. Ann Intern Med. 136: 747-757.

Hezode C, Castera L, Roudot-Thoraval F , Bouvier-Alias M, Rosa I, Roulot D, Leroy V, Mallat A, Pawlotsky JM (2011). Liver stiffness diminishes with antiviral response in chronic hepatitis C. Aliment Pharmacol Therap.34(6):656–663.

Hnatyszyn HJ (2005). Chronic hepatitis C and genotyping: the clinical significance of determining HCV genotypes. Antivir Ther. 10: 1-11.

Hoofnagle JH (1997). Hepatitis C: The clinical spectrum of disease. Hepatol. 26 (Suppl 1): 15S-20S.

Hoofnagle JH, Mullen KD, Jones DB, Rustgi V, Di Bisceglie A, Peters M, Waggoner JG, Park Y and Jones EA (1986). Treatment of chronic non-A, non-B hepatitis with recombinant human alpha interferon. A preliminary report. New Engl J Med. 15: 1575-1578.

Hope RG, Murphy DJ and McLauchlan J (2002). The domains required to direct core proteins of hepatitis C virus and GB virus-B to lipid droplets share common features with plant oleosin proteins. J Biol Chem. 277:4261-4270.

Hsu CS, Liu CH, Liu CJ, Wang CC, Chen CL, Lai MY, Chen PJ, Chen DS and Kao JH (2009). Association of lipid profiles with hepatitis C viral load in chronic hepatitis C patients with genotype 1 or 2 infection. Am J Gastroenterol. 104(3): 598-604.

Hsu SC, Chang MH, Chen DS, Hsu HC and Lee CY (1991). Non-A, non-B hepatitis in children: a clinical, histologic, and serologic study. J Med Virol. 35: 1-6.

http://blast.ncbi.nlm.nih.gov/Blast.cg.

http://genomics.jp/sidirect/

http://hcv.lanl.gov/content/sequence/TRANSLATE/translate.html

http://www.ebi.ac.uk/thornton-srv/software/PROCHECK/

Hu Z, Muroyama R, Kowatari N, Chang J, Omata M and Kato N (2009). Characteristic mutations in hepatitis virus core gene related to the occurrence of hepatocellular carcinoma. Cancer Sci. 100: 2465-2468.

Huang H, Sun F, Owen DM., Li W, Chen Y, Gale Jr M and Ye J (2007). Hepatitis C virus production by human hepatocytes dependent on assembly and secretion of very low-density lipoproteins. Proc Natl Acad Sci USA. 104: 5848-5853.

Husain A, Malik FA, Nagra H, Ehsan A, Ahmad Z and Abid M (2009). Frequency of different HCV genotypes in Faisalabad. APMC. 3(1): 19-22.

Idrees M and Riazuddin S (2008). Frequency distribution of hepatitis C virus genotypes in different geographical regions of Pakistan and their possible routes of transmission. BMC Infec Dis. 8: 69.

Idrees M, Lal A, Naseem M and Khalid M (2008a). High prevalence of hepatitis C virus infection in the largest province of Pakistan. J Dig Dis. 9: 95-103.

Ijaz T, Shahzad MK, Sarfraz N and Khan MA (2008b). Prevalence of Genotype 3a Hepatitis C Virus (HCV) In the Infected Population of Lahore, Pakistan. IJAVMS. 2: 14-17.

Imbert-Bismut F, Ratziu V, Pieroni L, Charlotte F, Benhamou Y and Poynard T; MULTIVIRC Group (2001). Biochemical markers of liver fibrosis in patients with hepatitis C virus infection: a prospective study. Lancet. 357: 1069-1075.

Inamullah, Idrees M, Ahmed H, Sajid-ul-Ghafoor, Ali M, Ali L and Ahmed A (2011). Hepatitis C virus genotypes circulating in district Swat of Khyber Pakhtoonkhaw, Pakistan. Virol J. 8: 16.

Iqbal S, Ahmed R, Yousaf MH, Mumtaz A, Amin D, Rasool G and Manzoor A (2007). HCV infected patients; assessment of major genotypes and subtypes of hepatitis C virus. Profession Med J. 14(2): 266-271.

Ishak K, Baptista A, Bianchi L, Callea F, Groote JD, Gudat F, Denk H, Desmet V Korb G, MacSween RNM, Phillips MJ, Portmann BG, Poulsen H, Scheuer J, Schmid M and Thaler H (1995). Histological grading and staging of chronic hepatitis. J Hepatol. 22(6): 696-9.

Itakura J, Nagayama K, Enomoto N, Hamano K, Sakamoto N, Fanning LJ, Kenny-Walsh E, Shanahan F and Watanabe M (2005). Viral load change and sequential evolution of entire hepatitis C virus genome in Irish recipients of single source-contaminated anti-D immunoglobulin. J Viral Hepat. 12:594–603.

Ito T and Lai MC (1997). Determination of the secondary structure of and cellular protein binding to the 3'-untranslated region of hepatitis C virus RNA genome. J Virol. 71: 8698-8706.

Jaeckel E, Cornberg M, Wedemeyer H, Santantonio T, Mayer J, Zankel M , Pastore G, Dietrich M, Trautwein C and Manns MP (2001).Treatment of acute hepatitis C with interferon alpha- 2b. N Engl J Med. 345: 1452-1457.

Jahan S, Khaliq S, Samreen B, Ijaz B, Khan M, Ahmad W, Ashfaq U and Hassan S (2011). Role of HCV Core gene of genotype 1a and 3a and host gene Cox-2 in HCV-induced pathogenesis. J Virol. 8: 295.

Jarmay K, Karacsony G, Nagy A and Schaff Z (2005). Changes in lipid metabolism in chronic hepatitis C. W J Gastroenterol. 11: 6422-6428.

Jeannel D, Fretz C, Traore Y, Kohdjo N, Bigot A, Gamy EP, Jourdan G, Kourouma K, Maertens G, Fuloux F, Fournel JJ and Stuyver L (1998). Evidence for high genetic diversity and long-term endemicity of hepatitis C virus genotypes 1 and 2 in West Africa. J Med Virol. 55: 92-97.

Jimenez-Hernandez N, Torres-Puente M, Bracho MA, Garcia-Robles I, Ortega E, Olmo J del, Carnicer F, Gonzalez-Candelas F and Moya A (2007). Epidemic dynamics of two coexisting hepatitis C virus subtypes. J Gen Virol. 88: 123-133.

Johnston DE (1999). Special considerations in interpreting liver function tests. Am Fam Physician. 59: 2223–30.

Jules LD and Isselbacher KJ (2001). Acute viral hepatitis. In: Braunwald E, Fauci AS, Kasper DL, et al (eds). Harrison's principals of internal medicines.15th Edition. 2: 1721-1737.

Kabir A, Alavian SM and Keyvani H (2006). Distribution of hepatitis C virus in patients infected by different sources and its correlation with clinical and virological parameters: A preliminary study. Compar Hepatol. 5: 4-9.

Kaiser S, Hass H and Gregor M (2005). Consensus Interferon in combination with gamma interferon and/or ribavirin leads to a significant response in treatment-naïve hepatitis C genotype 1 patients. Abstract 66839. 56th Annual Meeting of the American Association for the Study of Liver Diseases (56th AASLD), San Francisco, CA.

Kakumu S, Aiyama T, Okumura A, Iwata K, Ishikawa T and Yoshioka K (1997). Earlier loss of hepatitis C virus RNA in interferon therapy can predict a long-term response in chronic hepatitis C. J Gastroenterol Hepatol. 12(6): 468-72.

Kamal SM (2011). Hepatitis C virus genotype 4 therapy: progress and challenges. Liver Int. 1: 45-52.

Kaneko Y, Takada T and Kawano S (1999). Population biology of Aesculusturbinata Blume: A demographic analysis using transition matrices on a natural population along a riparian environmental gradient. Plant Species Biol 14:47-68.

Kanto T, Hayashi N, Takehara T, Hagiwara H, Mita E, Naito M, Kasahara A, Fusamoto H and Kamada T (1994). Buoyant density of hepatitis C virus recovered from infected hosts: two different features in sucrose equilibrium density-gradient centrifugation related to degree of liver inflammation. Hepatol. 19: 296-302.

Kantzanou M, Lucas M, Barnes E, Komatsu H, Dusheiko G, Ward S, Harcourt G and Klenerman P (2003). Viral escape and T cell exhaustion in hepatitis C virus infection analysed using class I peptide tetramers. Immunol Lett. 85: 165-171.

Kao JH (2002). Hepatitis B viral genotypes: clinical relevance and molecular characteristics. J Gastroenterol Hepatol. 17: 643-650.

Kato N, Sekiya H, Ootsuyama Y, Nakazawa T, Hijikata M, Ohkoshi S and Shimotohno K (1993). Humoral immune response to hypervariable region 1 of the putative envelope glycoprotein (gp70) of hepatitis C virus. J Virol. 67(7): 3923–3930.

Kenny-Walsh E (1999). Clinical outcomes after hepatitis C infection from contaminated anti-D immune globulin. Irish Hepatology Research Group. N Engl J Med. 340(16): 1228–1233.

Khan H and Zarif M (2006). Risk factors, complications and prognosis of cirrhosis in a tertiary care hospital of peshawer. Hepat Monthly. 6(1):7-10.

Khan N, Tanaka Y, Azam Z, Abbas Z, Kurbanov F, Saleem U, Hamid S, Jafri W and Mizokami M (2009). Epidemic spread of hepatitis C virus genotype 3a and relation to high incidence of hepatocellular carcinoma in Pakistan. J Med Virol. 18(7): 1189-1197.

Khan TS, Rizvi F and Rashid A (2003). Hepatitis C seropositivity among liver disease patients in Hazara, Pakistan. J Ayub Med Coll Abbottabad. 15(2): 53-55.

Khokhar N, Asif N and Khokhar OS (2003). Serotype 3 is most common hepatitis C serotype in Pakistan. However, significant numbers are untypeable. Hepatol. 38(1): 271-272.

Kim JL, Morgenstern KA, Griffith JP, Dwyer MD, Thomson JA, Murcko MA, Lin C and Caron PR (1998). Hepatitis C virus NS3 RNA helicase domain with a bound oligonucleotide: The crystal structure provides insights into the mode of unwinding. Struc. 6: 89-100.

Kim WR, Flamm SL, Di Bisceglie AM, Bodenheimer HC and Public Policy Committee of the American Association for the Study of Liver Disease (2008). Serum activity of alanine aminotransferase (ALT) as an indicator of health and disease. Hepatol. 47: 1363-1370.

Knodell RG, Ishak KG, Black WC, Chen TS, Craig R, Kaplowitz N, Kiernan TW and Wollman J (1981). Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. Hepatol. 1(5): 431-5.

Kobayashi M, Ikeda K, Matsuda M, Koike H, Hashimoto M and Kumada H (1999). Predictive value of different hepatitis C serological assays in the treatment of chronic hepatitis C with interferon a. J Gastroenterol. 34(1): 94-99.

Kotenko SV, Gallagher G, Baurin VV, Lewis-Antes A, Shen M, Shah NK, Langer JA, Sheikh F, Dickensheets H and Donnelly RP (2003). IFN-lambdas mediate

antiviral protection through a distinct class II cytokine receptor complex. Nat Immunol. 4:69-77.

Kuntz E and Kuntz HD (2006). Hepatology principles and practices. History, Morphology, Biochemistry, Diagnostics, Clinical therapy. Springer, 2nd Edition.p: 413.

Kuntz E and Kuntz HD (2008). Clinical aspects of liver diseases. (A-SEN). In: Hepatology Textbook and Atlas. History, Molecular biology, Diagnostics, Clin Therap. 419-471.

Lackner C, Struber G, Liegl B, Leibl S, Ofner P, Bankuti C, Bauer B and Stauber RE (2005). Comparison and validation of simple noninvasive tests for prediction of fibrosis in chronic hepatitis C. Hepatol. 41: 1376–1382.

Land A and Braakman I (2001). Folding of the humanimmunode?ciency virus type 1 envelope glycoprotein in theendoplasmic reticulum. Biochimie. 83: 783-790.

Lange CM, Sarrazin C and Zeuzem S (2010). Specifically targeted anti-viral therapy for hepatitis C - a new era in therapy. Aliment Pharmacol Ther. 32:14-28.

Large MK, Kittlesen DJ and Hahn YS (1999). Suppression of host immune response by the core protein of hepatitis C virus: possible implications for hepatitis C virus persistence. J Immunol. 162: 931-938.

Lau DT, Kleiner DE, Ghany MG, Park Y, Schmid P and Hoofnagle JH (1998). 10-year follow-up after interferon-alpha therapy for chronic hepatitis C. Hepatol. 28: 1121-1127.

Lauer GM and Walker BD (2001). Hepatitis C virus infection. N Engl J Med. 345: 41-52.

Lavanchy D (2011). Evolving epidemiology of hepatitis C virus. Clinic Microbiol Infec. 17(2): 107-115.

Layden JE and Layden TJ (2002).Viral kinetics of hepatitis C: new insights and remaining limitations. Hepatol. 35: 967–970.

Le Guillou-Guillemette H, Vallet S, Gaudy-Graffin C, Payan C, Pivert A, Goudeau A and Lunel-Fabiani F (2007). Genetic diversity of the hepatitis C virus: impact and issues in the antiviral therapy. W J Gastroenterol. 13(17): 2416-2426.

Le Pogam S, Seshaadri A, Kosaka A, Chiu S, Kang H, Hu S, Rajyaguru S, Symons J, Cammack N and Na'jera I (2008). Existence of hepatitis C virus NS5B variants naturally resistant to non-nucleoside, but not to nucleoside, polymerase inhibitors among untreated patients. J Antimicrob Chemother. 61:1205–1216.

Lee SB, Bablanian R and Esteban M (1996). Regulated expression of the interferon-induced protein kinase p68 (PKR) by vaccinia virus recombinants inhibits the replication of vesicular stomatitis virus but not that of poliovirus. J Interferon Cytokine Res. 16(12):1073-8.

Lerat H, Honda M, Beard MR, Loesch K, Sun J, Yang Y, Okuda M, Gosert R, Xiao SY, Weinman SA and Lemon SM (2002). Steatosis and liver cancer in transgenic mice expressing the structural and nonstructural proteins of hepatitis C virus. Gastroenterol. 122:352 -365.

Lévêque VJ and Wang Q (2002). RNA-dependent RNA polymerase encoded by hepatitis C virus: biomedical applications. Cell Mol Life Sci.59:909-919.

Li H, Hughes AL, Bano N, McArdle S, Livingston S, Deubner H, McMahon BJ, Townshend-Bulson L, McMahan R , Rosen HR and Gretch DR (2011). Genetic diversity of near genome-wide hepatitis c virus sequences during chronic infection: evidence for protein structural conservation over time. PLOS One. 6(5): 1-10.

Liew M, Erali M, Page S, Hillyard D and Wittwer C (2004). Hepatitis C Genotyping by Denaturing High-Performance Liquid Chromatography. J Clin Microbiol. 42(1): 158-163.

Lin C and Rice CM (1995). The hepatitis C virus NS3 serine proteinase and NS4A cofactor: establishment of a cell-free trans-processing assay. Proc Natl Acad Sci USA. 92(17): 7622–7626.

Lindenbach BD and Rice CM (2001). Flaviviridae: the viruses and their replication. In: Knipe DM and Howley PM (eds). Fields virology. Philadelphia: Lippincott Williams and Wilkins. 991-1042.

Lohmann V, Korner F, Herian U and Bartenschlager R (1997). Biochemical properties of Hepatitis C virus NS5B RNA-dependent RNA polymerase and identification of amino acid sequence motifs essential for enzymatic activity. J Virol. 71:8416-8428.

Lohmann V, Roos A, Korner F, Koch JO and Bartenschlager R (2000). Biochemical and structural analysis of the NS5B RNA-dependent RNA polymerase of the hepatitis C virus. J Viral Hepat. 7:167–174.

Luo G, Hamatake RK, Mathis DM, Racela J, Rigat KL, Lemm J and Colonno RJ (2000). De Novo initiation of RNA synthesis by the RNA-dependent RNA polymerase (NS5B) of hepatitis C virus. J Virol. 74: 851-863.

Ma H, Leveque V, De Witte A, Li W, Hendricks T, Clausen SM, Cammack N and Klumpp K (2005). Inhibition of native hepatitis C virus replicase by nucleotide and non-nucleoside inhibitors. Virol. 332:8–15.

Maggi G, Bottelli R, Gola D, Perricone G, Posca M, Zavaglia C and Ideo G (1996). Serum cholesterol and chronic hepatitis C. Ital J Gastroenterol. 8: 436-440.

Malavazi I, Abrao EP, Mikawa AY, Landgraf VO and da Costa PI (2004). Abnormalities in apolipoprotein and lipid levels in an HIV-infected Brazilian

population under different treatment profiles: the relevance of apolipoprotein E genotypes and immunological status. Clin Chem Lab Med. 42(5): 525-32.

Mangia A and Mottola L (2012). What's new in HCV genotype 2 treatment. Liv Int. 32 (1):135–140.

Mangia A, Cascavilla I, Lezzi G, Spirito F, Maertens G, Parlatore L, Saracco G, Rizzetto M and Andriulli A (1997). HCV genotypes in patients with liver disease of different stages andseverity. J Hepatol. 26(6): 1173-1178.

Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M and Albrecht JK (2001). Peginterferonalfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet. 358: 958-965.

Marcellin P, Asselah T and Boyer N (2002). Fibrosis and disease progression in hepatitis C. Hepatol. 36(5 Suppl 1): S47-56.

Marcellin P, Boyer N, Gervais A, Martinot M, Pouteau M, Castelnau C, Kilani A, Areias J, Auperin A, Benhamou JP, Degott C and Erlinger S (1997). 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. 127: 875-881.

Marcello T, Grakoui A, Barba-Spaeth G, Machlin ES, Kotenko SV, MacDonald MR and Rice CM (2006). Interferons alpha and lambda inhibit hepatitis C virus replication with distinct signal transduction and gene regulation kinetics. Gastroenterol. 131:1887-1898.

Martinot-Peignoux M, Boyer N, Pouteau M, Castelnau C, Giuily N, Duchatelle V, Aupérin A, Degott C, Benhamou JP, Erlinger S and Marcellin P (1998). Predictors of sustained response to alpha interferon therapy in chronic hepatitis C. J Hepatol. 29: 214-23.

Martinot-Peignoux M, Marcellin MC, Pouteau N, Castelnau M, Boyer C, Poliquin I, Degott V, Descombes L, Breton and Milotova V (1995). Pretreatment hepatitis C virus RNA levels and hepatitis C virus genotype are main and independent prognostic factors of sustained response to interferon alpha therapy in chronic hepatitis. Hepatol. 22: 1050-1056.

Martinot-Peignoux M, Yer N and Le-Breton V (2000). A new step towards standardization of serum hepatitis C virus RNA quantification in patients with chronic hepatitis C. Hepatol. 31: 726-729.

Marukian S, Jones CT, Andrus L, Evans MJ, Ritola KD, Charles ED, Rice CM and Dustin LB (2008). Cell culture-produced hepatitis C virus does not infect peripheral blood mononuclear cells. Hepatol. 48(6): 1843–1850.

Marzouk D , Sass J, Bakr I, El Hosseiny M, Abdel-Hamid M, Rekacewicz C, Chaturvedi N, Mohamed MK and Fontanet A (2007). Metabolic and cardiovascular risk profiles and hepatitis Cvirus infection in rural Egypt. Gut. 65: 1105-1110.

Masood Z, Jawaid M, Khan RA and Rehman S (2005). Screening for hepatitis B & C: A routine prospective investigation? Pak J Med Sci. 21: 455-459.

Mathews MB, Hershey JWB and Sonenberg N (1996). Translational Control NY.: Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

McGarvey MJ and Houghtom M (2005). Section V hepatitis C virus. Structure and molecular virology. In: Thomas HC, Lemon S and Zuckerman AJ (eds).viral hepatitis.3rd Edition. Blackwell publishing group p: 381.

McHutchinson J, Person JL and Govindarajan S (1992). Improved detection of Hepatitis C Virus antibodies in High-Risk population. Hepatol. 5: 19-25.

McHutchison JG, Bacon BR, Gordon SC, Lawitz E, Shiffman M, Afdhal NH, Jacobson IM,. Muir A, Vicari A, Efler S, Al-Adhami M, Morris ML and

Davis HL (2006). Final results of a multi-center phase 1b, randomized, placebo-controlled, dose-escalation trial of CPG 10101 in patients with chronic hepatitis C virus. J Hepatol. 44: S49.

McHutchison JG, Gordon SC, Schiff ER, Shiffman ML, Lee WM, Rustgi VK and Goodman ZD (1998). Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. New Eng J Med. 339: 1485-1492.

McHutchison JG, Poynard T, Davis GL, Esteban-Mur R, Harvey J, Ling M, Cort S, Fraud JJ, Albrecht J and Dienstag J (1999). Evaluation of hepatic HCV RNA before and after treatment with interferon alfa 2b or combined withribavirin in chronic hepatitits. Hepatol. 30(2): 363A.

McOmish F, Yap PL, Dow BC, Follett EC, Seed C, Keller AJ, Cobain TJ, Krusius T, Kolho E, Naukkarinen R, Lin C, Lai C, Leong S, Medgyesi GA, Hejjas M, Hiyokawa H, Fukada K, Cuypers T, Saeed AA, Al-Rasheed AM, Lin M and Simmonds P (1994). Geographical Distribution of Hepatitis C Virus Genotypes in Blood Donors: an International Collaborative Survey. J Clin Microbiol. 32(4): 884-892.

Mellor J, Holmes EC, Jarvis LM, Yap PL and Simmonds P (1995). Investigation of the pattern of hepatitis C virus sequence diversity in different geographical regions: implications for virus classification. J Gen Virol. 76: 2493-2507

Mellor J, Walsh EA, Prescott LE, Jarvis LM, Davidson F, Yap PL,Simmonds P, Nowlcki MJ, Mosley JW, Lin CK, Lai CL, Deolim G, Martins A, Ong YM, Teo D, Lin M, Nuchprayoon C and Tanprasert S (1996). Survey of type 6 group variants of hepatitis C virus in Southeast Asia by using a core-based genotyping assay. J Clin Microbiol. 34: 417-423.

Michalak JP, Wychowski C, Choukhi A, Meunier JC, Ung S, Rice CM and Dubuisson J (1997). Characterization of truncated forms of Hepatitis C virus glycoproteins. J Gen Virol. 78:2299-2306.

Miller JP (1990). Dyslipoproteinaemia of liver diseases. Baillieres Clin Endocrinol Metab. 4: 807-3.

Missiha SB, Ostrowski M and Heathcote EJ (2008). Disease progression in chronic hepatitis C: modifiable and nonmodifiable factors. Gastroenterol. 134(6): 1699–1714.

Mizokami M, Gojobori T, Ohba K, Ikeo K, Ge XM, Ohno T, Orito E and Lau JY (1996). Hepatitis C virus types 7, 8 and 9 should be classified as type 6 subtypes. J Hepatol. 24: 622-624.

Moatter T, Hussainy AS, Hamid S, Ahmad Z and Siddiqui S (2002). Comparative analysis of viral titers and histologic features of Pakistani patients infected with hepatitis C virus type 3. Int J Infect Dis. 6: 272-276.

Mondelli MU, Cerino A, Segagni L, Meola A, Cividini A, Silini E and Nicosia A (2001). Hypervariable region 1 of hepatitis C virus: immunological decoy or biologically relevant domain? Antiviral Res. 52(2): 153–159.

Moritani M, Adachi K, Arima N, Takashima T, Miyaoka Y, Niigaki M, Furuta K, Sato S and Kinoshita Y (2005). A study of arteriosclerosis in healthy subjects with HBV and HCV infection. J Gastroenterol. 40: 1049-1053.

Moriya K, Shintani Y, Fujie H, Miyoshi H, Tsutsumi T, Yotsuyanagi H, Iino S, Kimura S and Koike K (2003). Serum lipid profile of patients with genotype 1b hepatitis C viral infection in Japan. Hepatol Res. 25(4): 371-376.

Morrow MP, Pankhong P, Laddy DJ, Schoenly KA, Yan J, Cisper N and Weiner DB (2009). Comparative ability of IL-12 and IL-28B to regulate Treg populations and enhance adaptive cellular immunity. Blood. 113: 5868-5877.

Morrow MP, Yan J, Pankhong P, Shedlock DJ, Lewis MG, Talbott K, Toporovski R, Khan AS, Sardesai NY and Weiner DB (2010). IL-28B/IFN-lambda 3 drives granzyme B loading and significantly increases CTL killing activity in macaques. Mol Ther. 18:1714-1723.

Muhammad N and Jan MA (2005). Frequency of hepatitis "C" in Buner, NWFP. J Coll Physicians Surg Pak. 15: 11-14.

Muir AJ, Bornstein JD and Killenberg PG (2004). Peginterferon alfa-2b and ribavirin for the treatment of chronic hepatitis C in Blacks and non-Hispanic Whites. New Eng J Med. 350: 2265-2271.

Muir AJ, Shiffman ML, Zaman A, Yoffe B, de la Torre A, Flamm S, Gordon SC, Marotta P, Vierling JM, Lopez-Talavera JC, Byrnes-Blake K, Fontana D, Freeman J,Gray T, Hausman D, Hunder NN and Lawitz E (2010). Phase 1b study of pegylated interferon lambda 1 with or without ribavirin in patients with chronic genotype 1 hepatitis C virus infection. Hepatol. 52:822-832.

Murakami S, Okubo K, Tsuji Y, Sakata H, Takahashi T, Kikuchi M and Hirayama R (2004). Changes in liver enzymes after surgery in anti-hepatitis C virus-positive patients. World J Surg.28: 671-674.

Nagata S, Taira H, Hall A, Johnsrud L, Streuli M, Ecsödi J, Boll W, Cantell K and Weissmann C (1980). Synthesis in E. coli of a polypeptide with human leukocyte interferon activity. Nature. 284: 316-320

Ndjomou J, Pybus OG and Matz B (2003). Phylogenetic analysis of hepatitis C virus isolates indicates a unique pattern of endemic infection in Cameroon. J Gen Virol. 84: 2333-2341.

Nei M and Kumar S (2000). Molecular Evolution and Phylogenetics. Oxford University Press, New York.

Nelson D, Benhamou Y, Chuang WL, Lawitz E, Flisiak R, Rodriguez-Torres M, Bain V, Patel K, Cronin PW, Pulkstenis E, Subramanian GM and McHutchison JG (2009). Efficacy and safety results of albinterferon alfa-2b in combination with ribavirin in interferon alfa treatment naive patients with genotype 2 or 3 chronic hepatitis. J Hepatol. 50S: 378.

Neumann AU, Lam NP, Dahari H, Davidian M, Wiley TE, Mika BP, Perelson AS and Layden TJ (2000). Differences in viral dynamics between genotypes 1 and 2 of hepatitis C virus. J Infect Dis. 182: 28-35.

Neumann AU, Lam NP, Dahari H, Gretch DR, Wiley TE, Layden TJ and Perelson AS (1998). Hepatitis C viral dynamics *in vivo* and the anti-viral efficacy of interferon-alpha therapy. Science. 282: 103-107.

Nguyen HA, Miller AI, Dieperink E, Willenbring ML, Tetrick LL, Durfee JM, Ewing SL and Ho SB (2002). Spectrum of disease in U.S. veteran patients with hepatitis C. Am J Gastroenterol. 97: 1813-1820.

Nguyen MH and Keeffe EB (2005). Prevalence and treatment of hepatitis C virus genotypes 4, 5, and 6. Clin Gastroenterol Hepatol. 3: S97–101.

Niederau C, Lange S, Heintges T, Erhardt A, Buschkamp M, Hurter D, Nawrocki M, Kruska I, Hensel F, Petry W and Haussinger D (1998). Prognosis of chronic Hepatitis C: results of a large prospective cohort study. Hepatol. 28: 1687-1695.

Nielsen SU, Bassendine MF, Burt AD, Martin C, Pumeechockchai W and Toms GL (2006). Association between hepatitis C virus and very-low-density lipoprotein (VLDL)/LDL analyzed in iodixanol density gradients. J Virol. 80(5): 2418-2428.

NIH (1997a). Consensus Development Conference Panel Statement: Management of hepatitis C. Hepatol. 26: 1906-1911.

NIH (1997b). Management of Hepatitis C. consensus Development Conference Statement.March24-26. http://consensus.nih.gov/1997/1997HepatitisC105html.htm.

Nolte FS, Thurmond C and Fried MW (1995). Preclinical evaluation of AMPLICOR hepatitis C virus test for detection of Hepatitis C virus RNA. J Clin Microbiol. 33: 1775-1778.

Noppornpanth S, Sablon E, Nys K, Lien T, Brouwer J, Brussel M, Smits S, Poovorawan Y, Osterhaus A and Haagmans B (2006). Genotyping hepatitis C virus from Southeast Asia by a novel line probe assay that simultaneously detects core and 5' untranslated regions. J Clin Microbiol. 44: 3969-3974.

Ohba K, Mizokami M, Ohno T, Suzuki K, Orito E, Ina Y, Lau JYN and Gojobori T (1995). Classification of hepatitis C virus into major types and subtypes based on molecular evolutionary analysis. Virus Res. 36: 201-214.

Okamoto H, Kurai K, Okada S, Yamamoto K, Lizuka H, Tanaka T, Fukuda S, Tsuda F and Mishiro S (1992). Full-length sequence of a hepatitis C virus genome having poor homology to reported isolates: comparative study of four distinct genotypes. Virol. 188: 331-341.

Okamoto H, Okada S, Sugiyama Y, Kurai K, Iizuka H, Machida A, Miyakawa Y and Mayumi M (1991). Nucleotide sequence of the genomic RNA of hepatitis C virus isolated from a human carrier: comparison with reported isolates for conserved and divergent regions. J Gen Virol. 72: 2697-2704.

Ooi K, Shiraki K, Sakurai Y, Morishita Y and Nobori T (2005). Clinical significance of abnormal lipoprotein patterns in liver diseases. Int J Mol Med. 15:655-660.

Oonk G (2007). Global Indian Diasporas: exploring trajectories of migration and theory (1st ed). University press, Armsterdam.

Otto GA, Lukavsky PJ, Lancaster AM, Sarnow P and Puglisi JD (2002). Ribosomal proteins mediate the hepatitis C virus IRES-HeLa 40S interaction. RNA. 8: 913-923.

Pawlotsky JM (2003). Hepatitis C virus genetic variability: pathogenic and clinical implications. Clin Liver Dis. 7: 45–66.

Pawlotsky JM (2006). Therapy of hepatitis C: from empiricism to cure. Hepatol. 43(Suppl 1):S207–S220.

Pawlotsky JM (2009). Therapeutic implications of hepatitis C virus resistance to antiviral drug. Therap Advance Gastroenterol. 2(4):205-219.

Pawlotsky JM and McHutchison JG (2004). Hepatitis C. Development of new drugs and clinical trials: promises and pitfalls. Summary of an AASLD hepatitis single topic conference, Chicago, IL, February 27–March 1, 2003. Hepatol. 39:554–567.

Pawlotsky JM, Tsakiris L, Roudotthoraval F, Pellet C, Stuyver L, Duval J and Dhumeaux D (1995). Relationship between Hepatitis-C Virus Genotypes and Sources of Infection in Patients with Chronic Hepatitis-C. J Infect Dis. 171(6): 1607-1610.

Pazienza V, Vinciguerra M, Andriulli A and Mangia A (2010). Hepatitis C Virus core protein genotype 3a increases SOCS-7 expression through PPAR-{gamma} in Huh-7 cells. J Gen Virol. 91: 1678-1686.

Peck-Radosavljevic M (2007). Coagulation disorders in chronic liver disease. Aliment Pharmacol Ther. 1:21-8.

Penin F, Brass V, Appel N, Ramboarina S, Montserret R, Ficheux D, Blum HE, Bartenschlager R and Moradpour D (2004a). Structure and function of the membrane anchor domain of hepatitis C virus nonstructural protein 5A. J Biol Chem. 279: 40835-40843.

Penin F, Combet C, Germanidis G, Frainais PO, Deleage G and Pawlotsky JM (2001). Conservation of the conformation and positive charges of hepatitis C virus E2 envelope glycoprotein hypervariable region 1 points to a role in cell attachment. J Virol. 75: 5703-5710.

Penin F, Dubuisson J, Rey FA, Moradpour D and Pawlotsky JM (2004b). Structural Biology of Hepatitis C Virus. Hepatol. 39(1): 5–19.

Perlemuter G, Sabile A, Letteron P, Topilco A, Chretien Y, Koike K, Pessayre D Chapman J, Barba G and Brechot C (2002). Hepatitis C virus core protein inhibits microsomal triglyceride transfer protein activity and very low density lipoprotein secretion: a model of viral-related steatosis. FASEB J. 16 (2): 185-194.

Petrik J, Parker H and Alexander GJM (1999). Human hepatic glyceraldehyde-3-phosphate dehydrogenase binds to the poly (U) tract of the 3' Non-Coding region of the hepatitis C virus genomic RNA. J Gen Virol. 80: 3109-3113.

Pileri P, Uematsu Y, Campagnoli S, Galli G, Falugi F, Petracca R, Weiner AJ, Houghton M, Rosa D, Grandi G and Abrignani S (1998). Binding of hepatitis C virus to CD81. Science. 282: 938-941.

Pilli M, Zerbini A, Penna A, Orlandini A, Lukasiewicz E, Pawlotsky JM, Zeuzem S, Schalm SW, von Wagner M, Germanidis G, Lurie Y, Esteban JI, Haagmans BL,Hezode C, Lagging M, Negro F, Homburger Y, Neumann AU, Ferrari C and Missale G; DITTO-HCV Study Group (2007). HCV-specific T-cell response in relation to viral kinetics and treatment outcome (DITTO-HCV project). Gastroenterol. 133:1132-1143.

Ploss A and Dubuisson J (2012). New advances in the molecular biology of hepatitis C virus infection: towards the identification of new treatment targets. Gut. 61(Suppl 1):i25–i35

Poynard T, Bedossa P and Opolon P (1997). Natural history of liver fibrosis progression in patients with chronic hepatitis C. The OBSVIRC, METAVIR, CLINIVIR, and DOSVIRC groups. Lancet.349825–832.832

Poynard T, Bedossa P, Chevallier M, Matturin P, Lemonnier C and Trepo C (1995). A comparison of three interferon alpha 2b regimens for long terms treatment of chronic non-A, non-B hepatitis. New Eng J Med. 332: 1457-1462.

Poynard T, Marcellin P, Lee SS, Niederau C, Minuk GS, Ideo G and Bain V (1998). Randomized trial of interferon alpha 2b plus ribavirin for 48 weeks or for 24 weeks versus interferon alpha 2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. International Hepatitis Interventional Therapy Group (IHIT). Lancet. 352: 1426-1432.

Poynard T, Yuen MF, Ratziu V and Lai CL (2003). Viral hepatitis C. Lancet. 362:2095-2100.

Puig M, Major ME, Mihalik K and Feinstone SM (2004). Immunization of chimpanzees with an envelope protein -based vaccine enhances specific humoral and cellular immune response that delay hepatitis C infection. Vaccine.24: 991-1000.

Purcell RH (1994). Hepatitis C virus: Historical perspective and current concept. FEMS Microbiol Rev.14: 181-191.

Pybus OG, Barnes E, Taggart R, Lemey P, Markov PV, Rasachak B, Syhavong B, Phetsouvanah R, Sheridan I, Humphreys IS, Lu L, Newton PN and Klenerman P (2009). Genetic History of Hepatitis C Virus in East Asia. J Virol. 83(2): 1071-1082.

Pybus OG, Markov PV, Wu A and Tatem AJ (2007). Investigating the endemic transmission of the hepatitis C virus. Int J Parasitol. 37: 839-849.

Qazi MA, Fayyaz M, Chaudhary GMD, Jamil A, Malik AH, Gardezi AI and Bukhari MH (2006). Hepatitis C virus genotypes in Bahawalpur. Biomedica. 22: 51-54.

Quarleri JF, Robertson BH, Mathet VL, Feld M, Espínola L, Requeijo MP, Mandó O, Carballal G and Oubiña JR (2000). Genomic and phylogenetic analysis of hepatitis C virus isolates from argentine patients:a six-year retrospective study. J Clin Microbiol. 38: 4560-4568.

Ramcharran D, Wahed AS, Conjeevaram HS, Evans RW, Wang T, Belle SH and Yee LJ (2011). Serum lipids and their associations with viral levels and liver disease severity in a treatment-naive chronic hepatitis C type 1-infected cohort. J Viral Hepat. 18: 144-152.

Rauch A, Kutalik Z, Descombes P, Cai T, Di Iulio J, Mueller T, Bochud M, Battegay M, Bernasconi E, Borovicka J, Colombo S, Cerny A, Dufour JF, Furrer H,Günthard HF, Heim M, Hirschel B, Malinverni R, Moradpour D, Müllhaupt B, Witteck A, Beckmann JS, Berg T, Bergmann S, Negro F, Telenti A, Bochud PY and Swiss Hepatitis C Cohort Study; Swiss HIV Cohort Study (2010). Genetic variation in IL28B is associated with chronic hepatitis C and treatment failure: a genome-wide association study. Gastroenterol. 138:1338-1345.

Ray SC, Wang YM, Laeyendecker O, Ticehurst JR, Villano SA, Thomas DL Ray SC, Wang YM, Laeyendecker O, Ticehurst JR, Villano SA and Thomas DL (1999). Acute hepatitis C virus structural gene sequences as predictors of persistent viremia: hypervariable region 1 as a decoy. J Virol. 73(4): 2938–2946.

Raymond E, Boige V, Faivre S, Sanderink GJ, Rixe O, Vernillet L, Jacques C, Gatineau M, Ducreux M and Armand JP (2002). Dosage adjustment and pharmacokinetic profile of irinotecan in cancer patients with hepatic dysfunction. J Clin Oncol. 20: 4303-4312.

Reddy KR, Messinger D, Popescu M and Hadziyannis SJ (2009). Peginterferon alpha-2a (40 kDa) and ribavirin: comparable rates of sustained virological response in sub-sets of older and younger HCV genotype 1 patients. J Viral Hepat. 16(10): 724-31.

Reddy KR, Wright TL, Pockros PJ, Shiffman M, Everson G, Reindollar R, Fried MW, Purdum PP, Jensen D, Smith C, Lee WM, Boyer TD, Lin A, Pedder S and De Pamphilis J (2001). Efficacy and safety of pegylated (40-kd) interferon a-2a compared with interferon a-2a in non-cirrhotic patients with chronic hepatitis C. Hepatol. 33(2): 433-438.

Reed KE and Rice CM (2000). Overview of hepatitis C virus genome structure, polyprotein processing, and protein properties. Curr Top Microbiol Immunol. 242:55-84.

Ridge JP, Di Rosa F and Matzinger P (1998). A conditioned dendritic cell can be a temporal bridge between a CD-41 T-helper and a T-Killer cell. Nature. 393: 474-478.

Robek MD, Boyd BS and Chisari FV (2005). Lambda interferon inhibits hepatitis B and C virus replication. J Virol. 79:3851-3854.

Robertson B, Myers G, Howard C, Brettin T, Bukh J, Gaschen B, Gojobori T, Maertens G, Mizokami M, Nainan O, Netesov S, Nishioka K, Shin i T, Simmonds P, Smith D, Stuyver L and Weiner A (1998). Classification, nomenclature, and database development for hepatitisC virus (HCV) and related viruses: proposals for standardization.International Committee on Taxonomy of Viruses. Arch Virol. 143: 2493-2503.

Ross R, Viazov S, Wolters B and Roggendorf M (2008). Towards a better resolution of hepatitis C virus variants: CLIP trademark sequencing of an HCV core fragment and automated assignment of genotypes and subtypes. J Virol Methods. 48: 25-33.

Roy A, Kucukural A and Zhang Y (2010). I-TASSER: a unified platform for automated protein structure and function prediction. Nature. 5: 725-738.

Ruggieri A, Argentini C, Kouruma F, Chionne P, Dugo E, Spada E, Dettori S, Sabbatani S and Rapicetta M (1996). Heterogeneity of hepatitis C virus genotype 2 variants in West Central Africa (Guinea Conakry). J Gen Virol. 77: 2073-2076.

Sahin I, Arabaci F, Sahin HA, Ilhan M, Ustun Y, Mercan R and Eminov L (2003). Does hepatitis C virus infection increase hematocrit and hemoglobin levels in hemodialyzed patients. Clin Nephrol. 60(6): 401-404.

Saito T, Hirai R, Loo YM, Owen D, Johnson CL, Sinha SC, Akira S, Fujita T and Gale Jr M (2007). Regulation of innate antiviral defenses through a shared repressor domain in RIG-I and LGP2. Proc Natl Acad Sci USA. 104: 582-7.

Sakaguchi M, Sato T and Groopman JE (1991). Human immunodeficiency virus infection of megakaryocytic cells. Blood. 77: 481-485.

Sakai T, Tanaka H, Shibata M, Suzuki W, Nomiya H, Kanazashi T, Lida S and Nakashizuka T (1999). Riparian disturbace and community structure of a Quercus-Ulmus forest in central Japan. Plant Ecol. 140: 99-109.

Samuel CE (2001). Antiviral actions of interferons. Clin Microbiol Rev 14(4): 778–809.

Santantonio T, Fasano M, Sinisi E, Guastadisegni A, Casalino C, Mazzola M, Francavilla R and Pastore G (2005). Efficacy of a 24-week course of PEG-interferon alpha-2b monotherapy in patients with acute hepatitis C after failure of spontaneous clearance. J Hepatol. 42: 329-33.

Sarrazin C, Teuber, G Kokka R, Rabenau H and Zeuzem S (2000). Detection of residual hepatitis C virus RNA by transcription-mediated amplification in

patients with complete virologic response according to polymerase chain reaction-based assays. Hepatol. 34(2): 818-823.

Sato K, Okamoto H, Aihara S, Hoshi Y, Tanaka T and Mishiro S (1993). Demonstration of sugar moiety on the surface of hepatitis C virions recovered from the circulation of infected humans. Virol. 196: 354-357.

Schroter M, Schafer P, Zollner B, Polywka S, Laufs R and Feucht HH (2001). Strategies for reliable diagnosis of hepatitis C infection: The need for a serological confirmatory assay. J Med Virol. 64 (3):320-324.

Seeff LB (1997). Natural history of hepatitis C. Hepatol. 26: 21S-28S.

Sen GC and Ransohoff RM (1993). Interferon-induced antiviral actions and their regulation. Adv Virus Res. 42: 57-102.

Senzolo M, Coppell J, Cholongitas E, Riddell A, Triantos CK, Perry D and Burroughs AK (2007). The effects of glycosaminoglycans on coagulation: a thromboelastographic study. Blood Coag Fibrinol. 18: 227.

Serfaty C, Andreani T and Giral P (2001). Hepatitis C virus induced hypobetalipoprotienemia: A possible mechanism for steatosis in chronic hepatitis C. J Hepatol. 34: 428-434.

Servoss J, Kitch D, Andersen J, Reisler R, Chung R, and Robbins G (2006). Predictors of antiretroviral-related hepatotoxicity in the adult AIDS Clinical Trial Group (1989–1999). J Acquir Immune Defic Syndr. 43: 320-323.

Sezaki H, Suzuki F, Kawamura Y, Yatsuji H, Hosaka T, Akuta N, Kobayashi M, Suzuki Y, Saitoh S, Arase Y, Ikeda K, Miyakawa Y and Kumada H (2009). Poor response to pegylated interferon and ribavirin in older women infected with hepatitis C virus of genotype 1b in high viral loads. Dig Dis Sci. 54(6): 1317-24.

Shepard CW, Finelli L and Alter MJ (2005). Global epidemiology of hepatitis C virus infection. Lanc Infect Dis. 5: 558-567.

Sheppard P, Kindsvogel W, Xu W, Henderson K, Schlutsmeyer S, Whitmore TE, Kuestner R, Garrigues U, Birks C, Roraback J, Ostrander C, Dong D, Shin J,Presnell S, Fox B, Haldeman B, Cooper E, Taft D, Gilbert T, Grant FJ, Tackett M, Krivan W, McKnight G, Clegg C, Foster D and Klucher KM (2003). IL-28, IL-29 and their class II cytokine receptor IL-28R. Nat Immunol 4(1): 63–68.

Sheth SG, Flamm SL, Gordon FD and Chopra S (1998). AST/ALT ratio predicts cirrhosis in patients with chronic hepatitis C virus infection. Am J Gastroenterol. 93: 44-48.

Shiffman ML, Salvatore J, Hubbard S, Price A, Sterling RK, Stravitz RT, Luketic VA and Sanyal AJ (2007). Treatment of chronic hepatitis C virus genotype 1 with peginterferon, ribavirin, and epoetin alpha. Hepatol. 46(2): 371–379.

Shiina M and Rehermann B (2008). Cell culture-produced hepatitis C virus impairs plasmacytoid dendritic cell function. Hepatol. 47(2): 385–95.

Shimada Y, Kato T, Ogami K, Horie K, Kokubo A, Kudo Y, Maeda E, Sohma Y, Akahori H and Kawamura K (1995). Production of thrombopoietin (TPO) by rat hepatocytes and hepatoma cell lines. Exp Hematol. 23(13):1388-1396.

Shoukry NH, Cawthon AG and Walker CM (2004). Cell-mediated immunity and the outcome of hepatitis C virus infection. Ann Rev Microbiol. 58: 391-424.

Siagris D, Christofidou M, Theocharis GJ, Pagoni N, Papadimitriou C, Lekkou A, Thomopoulos K, Starakis I, Tsamandas AC and Labropoulou-Karatza C (2006). Serum lipid pattern in chronic hepatitis C: histological and virological correlations. J Viral Hepat. 13(1): 56-61.

Silini E, Bono F, Cividini A, Cerino A, Bruno S, Rossi S, Belloni G, Brugnetti B, Civardi E, Salvaneschi L and Mondelli MU (1995). Differential distribution of hepatitis C virus genotypes in patients with and without liver function abnormalities. Hepatol. 21(2): 285-290

Simmonds P (1995). Variability of hepatitis C virus. Hepatol. 21:570–583.

Simmonds P (1999). Viral heterogeneity of the hepatitis C virus. J Hepatol. 31(1): 54-60.

Simmonds P (2001): The origin and evolution of hepatitis viruses in humans. J Gen Virol. 82: 693-712.

Simmonds P (2004). Genetic diversity and evolution of hepatitis C virus-15 years on. J Gen Virol. 85:3173-3188.

Simmonds P, Alberti A, Alter HJ, Bonino F, Bradley DW, Brechot C, Brouwer JT, Chan SW, Chayama K, Chen DS, Choo QL, Colombo M, Cuypers HTM, Date T, Dusheiko GM and Esteban JI (1994a). A proposed system for the nomenclature of hepatitis C viral genotypes. Hepatol. 19: 1321-1324.

Simmonds P, Bukh J, Combet C, Deléage G, Enomoto N, Feinstone S, Halfon P, Inchauspé G, Kuiken C, Maertens G, Mizokami M, Murphy DG, Okamoto H,Pawlotsky JM, Penin F, Sablon E, Shin-I T, Stuyver LJ, Thiel HJ, Viazov S, Weiner AJ and Widell A (2005). Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. Hepatol. 42: 962-973.

Simmonds P, Holmes EC, Cha TA, Chan SW, Hc Omish F, Irvine B, Beall E, Yap PL, Kolberg J and Urdea HS (1993). 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. 74: 2391-2399.

Simmonds P, Mellor J, Sakuldamrongpanich T, Nuchaprayoon C, Tanprasert S, Holmes EC and Smith DB (1996). Evolutionary analysis of variants of

hepatitis C virus found in South-East Asia: comparison with classifications based upon sequence similarity. J Gen Virol. 77(12): 3013-3024.

Simmonds P, Smith DB, McOmish F, Yap PL, Kolberg J, Urdea MS and Holmes EC (1994b). Identification of genotypes of hepatitis C virus by sequence comparisons in the core, E1 and NS-5 regions. J Gen Virol. 75(5):1053-61.

Slater-Handshy T, Droll DA, Fan X, Di Bisceglie AM and Chambers TJ (2004). HCV E2 glycoprotein: mutagenesis of N-linked glycosylation sites and its effects on E2 expression and processing. Virol. 319:36-48

Smith DB, Mellor J, Jarvis LM, Davidson F, Kolberg J, Urdea MS, Yap PL, Simmonds P and the International HCV Collaborative Study Group (1995). Variation of the hepatitis C virus 5_ non-coding region: implications for secondary structure, virus detection and typing. J Gen Virol. 76:1749–1761

Smith DB, Pathirana S, Davidson F, Lawlor E, Power J, Yap PL and Simmonds P (1997). The origin of hepatitis C virus genotypes. J Gen Virol. 78: 321–328.

Sobesky R, Lebray P, Nalpas B, Vallet-Pichard A, Fontaine H, Lagneau JL and Pol S (2008). Pathological evolution of hepatitis C virus "Healthy carriers". W J Gastroenterol. 14(24): 3861-3865.

Sommereyns C, Paul S, Staeheli P and Michiels T (2008). IFN-lambda (IFN-lambda) is expressed in a tissue-dependent fashion and primarily acts on epithelial cells in vivo. PLoS Pathog. 4(3): e1000017.

Spahn CMT, Penczek PA, Leith A and Frank J (2000). A method for differentiating proteins from nucleic acids in intermediate-resolution density maps: cryo-electron microscopy defines the quaternary structure of the Escherichia coli 70S ribosome. Str Fold Des. 8: 937-948.

Spann KM, Tran KC and Collins PL (2005). Effects of nonstructural proteins NS1 and NS2 of human respiratory syncytial virus on interferon regulatory factor 3, NF-kappaB, and proinflammatory cytokines. J Virol. 79(9): 5353–5362.

Stark GR, Kerr IM, Williams BR, Silverman RH and Schreiber RD (1998). How cells respond to interferons. Annu Rev Biochem 67: 227–264.

Strader DB, Wright T, Thomas DL and Seeff LB (2004). Diagnosis, management, and treatment of hepatitis C. Hepatol. 39: 1147–1171.

Strathdee SA, Zafar T, Brahmbhatt H, Baksh A and Hassan SU (2003). Rise in needle sharing among injection drug users in Pakistan during the Afghanistan war. Drug Alcohol Depend. 71(1):17-24.

Stumpf MPH and Pybus OG (2002). Genetic diversity and models of viral evolution for the hepatitis C virus. FEMS. Microbiol Lett. 214:143-152.

Sulkowski MS, Ray SC and Thomas DL (2002). Needle sticks transmission of hepatitis C. JAMA. 287(18): 2406-2413.

Sullivan DG, Bruden D, Deubner H, McArdle S, Chung M,1 Christensen C, Hennessy T, Homan C, Williams J, McMahon BJ and Gretch DR (2007). Hepatitis C Virus Dynamics during Natural Infection Are Associated with Long-Term Histological Outcome of Chronic Hepatitis C Disease. J Infect Dis. 196(2):239-48

Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, Bassendine M, Spengler U, Dore GJ, Powell E, Riordan S, Sheridan D, Smedile A,Fragomeli V, Müller T, Bahlo M, Stewart GJ, Booth DR and George J (2009). IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet. 41:1100-1104.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M and Kumar S (2011). MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood,

Evolutionary Distance, and Maximum Parsimony Methods. Mol Biol Evol. .28(10):2731-9.

Tan SL (2006). Hepatitis C Viruses: Genomes and Molecular Biology. Norfolk (UK): Horizon Bioscience.

Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, Nakagawa M, Korenaga M, Hino K, Hige S, Ito Y, Mita E, Tanaka E, Mochida S, Murawaki Y, Honda M, Sakai A, Hiasa Y, Nishiguchi S, Koike A, Sakaida I, Imamura M, Ito K, Yano K, Masaki N, Sugauchi F, Izumi N, Tokunaga K and Mizokami M (2009). Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet. 41:1105-1109.

Taniguchi S, Okamoto H, Sakamoto M, Kojima M, Tsuda F, Tanaka T, Munekata E, Muchmore EE, Peterson DA and Mishiro S (1993). A structurally ?exible and antigenically variable N-terminal domain of the hepatitis C virus E2/NS1 protein: implication for an escape from antibody. Virol. 195(1): 297–301.

Targher G, Bertolini L, Padovani R, Rodella S, Arcaro G and Day C (2007). Differences and similarities in early atherosclerosis between patients with non-alcoholic steatohepatitis andchronic hepatitis B and C. J Hepatol. 46(6): 1126– 1132.

Tataurov AY, You Y and Owczarzy R (2008). Predicting ultraviolet spectrum of single stranded and double stranded deoxiribonuclic acids. Biophys Chem. 133(1-3): 66-70

Abbas SZ, Al M, Muhammad AH, Shaw S and Abba SQ (2009). Frequency of HCV infection and its genotypes among patients attending a liver clinic and voluntary blood donors in a rural area of Pakistan. Pak J Med Sci. 25 (4):579-582.

Tellinghuisen TL and Rice CM (2002). Interaction between hepatitis C virus proteins and host cell factors. Curr Opin Microbiol. 5:419-427.

Thio CL, Thomas DL and Carrington M (2000). Chronic viral hepatitis and the human genome. Hepatol. 31(4): 819–827.

Thomas DL, Thio CL, Martin MP, Qi Y, Ge D, O'Huigin C, Kidd J, Kidd K, Khakoo SI, Alexander G, Goedert JJ, Kirk GD, Donfield SM, Rosen HR, Tobler LH,Busch MP, McHutchison JG, Goldstein DB, Carrington M (2009). Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. Nature. 461:798-801

Trepo C (1994). Seminar on hepatitis C. European Commission Public Health Unit 1994.

Tsang PS, Trinh H, Garcia RT, Phan JT, Ha NB, Nguyen H, Nguyen K, Keeffe EB and Nguyen MH (2008). Significant prevalence of histologic disease in patients with chronic hepatitis B and mildly elevated serum alanine aminotransferase levels. Clin Gastroenterol Hepatol. 6(5): 569-574.

Tsuchihara K, Tanaka T, Hijikata M, Kuge S, Toyoda H, Nomoto A, Yamamoto N and Shimotohno K (1997). Specific interaction of polypyrimidine tract-binding protein with the extreme 3' terminal structure of the hepatitis C virus genome. 3'X. J Virol. 71: 6720-6726.

Tsukiyama-kohara K, IizukaN, KoharaM and Nomoto A (1992). Internal ribosome entery site with in hepatitis C virus RNA. J Virol. 66: 1476-1483.

Ui-Tei K, Naito Y, Takashashi F, Haraguchi T, Ohki-Hamazaki H, Juni A, Ueda R and Saigo K (2004). Guidelines for the selection of highly effective siRNA sequences for mammalian and chick RNA interference. Nucleic Acid Res. 32(3): 936-948.

Vigerust DJ and. Shepherd VL (2007). Virus glycosylation: role in virulence and immune interactions. Trends Microbiol.5(5): 211-218.

Von Wagner M, Huber M and Berg T (2005). Peginterferon-alpha-2a (40KD) and ribavirin for 16 or 24 weeks in patients with genotype 2 or 3 chronic hepatitis C. Gastroenterol. 129: 522-7.

Vrolijk JM, de Knegt RJ, Veldt BJ, Orlent H and Schalm SW (2004). The treatment of hepatitis C: history, presence and future. Neth J Med. 62: 76-82.

Waggoner SN, Hall CHT and Hahn YS (2007). HCV core protein interaction with gC1q receptor inhibits Th1 differentiation of CD4 (+) T cells via suppression of dendritic cell IL-12 production. J Leukoc Biol. 82(6):1407-1419.

Wai CT, Greenson JK, Fontana RJ, Kalbfleisch JD, Marrero JA, Conjeevaram HS and Lok AS (2003). A simple noninvasive index can predict both significant fibrosis and cirrhosis in patients with chronic hepatitis C. Hepatol. 38: 518-52.

Walewski JL, Keller TR, Stump DD and Branch AD (2002). Evidence for a new hepatitis C virus antigen encoded in an overlapping reading frame. RNA. 7: 710-721.

Wang CS, Yao WJ, Wang ST, Chang TT and Chou P (2004). Strong association of hepatitis C virus (HCV) infection and thrombocytopenia: implications from a survey of a community with hyperendemic HCV infection. Clin Infect Dis. 39: 790–796.

Wansbrough-Jones MH, Frimpong E, Cant B, Harris K, Evans MRW and Teo CG (1998). Prevalence and genotype of hepatitis C virus infection in pregnant women and blood donors in Ghana. Trans Roy Soc Trop Med Hyg. 92: 496-499.