Qualitative Expression Analysis of Interferon Alpha Receptor in HCV Patients Resistant to Interferon Therapy

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(2015)

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By

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(2009-NUST-DirPhD-V&I-06)

A thesis submitted in partial fulfillment
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In

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DECLARATION

I hereby declare that the material contained in this thesis is my original work. I have not previously presented any part of this work elsewhere for any other degree.

Date: 27-08-2015 **Qudsia Bashir**

Certificate by Supervisors

Certified that contents and form of this thesis titled **"Qualitative Expression Analysis of Interferon Alpha Receptor in HCV Patients Resistant to Interferon Therapy"** submitted by Qudsia Bashir have been found satisfactory for the requirement of the award of Ph.D. degree.

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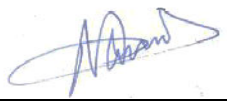
Member: 

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بسم الله الرحمن الرحيم

Dedicated

to

My Dear Family Members Especially
My Loving Parents and
Beloved Husband

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All Praise be to Almighty Allah, the most gracious and merciful, who always remained beneficent to me at every step to reach this destination. It is Allah to whom all the praises are attributed; the kind, the merciful, the compassionate whose blessings conferred me opportunity to gain knowledge, wisdom, guidance, health and renowned teachers to approach this destination successfully. All salutations are upon The Holy prophet MUHAMMAD (Salalla-ho-alaihe wasallam) who ordained every Muslim to seek knowledge from cradle to grave. My courage, confidence and success lies in my strong belief that relates me to the Holy Five People of the House.

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Qudsia Bashir

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LIST OF ABBREVIATIONS

ALT	Alanine transaminase
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
BMI	Body mass index
DNA	Deoxyribonucleic acid
cDNA	Coding deoxyribonucleic acid
DCs	Dendritic cells
ELISA	Enzyme linked immunosorbent assay
E1/E2	Envelope protein
H & E	Hematoxylin and eosin
HRQOL	Health-related quality of life
HAV	Hepatitis A virus
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HDV	Hepatitis D virus
HEV	Hepatitis E virus
HIV	Human Immunodeficiency Virus
IRF9	Interferon regulatory factor 9
IDU	Injection drug users
IRES	Internal Ribosomal Entry Site
IFNs	Interferons
IFN-α	Interferon alpha
IFNβ	Interferon beta
IFNω	Interferon omega
IFNγ	Interferon gamma
IFNAR1	Interferon alpha Receptor 1
IFNAR2	Interferon alpha Receptor 2

IgM	Immunoglobulin M
ISGs	IFN-stimulated genes
ISGF3	Interferon-sensitive gene factor-3
ISGs	Interferon-sensitive genes
ISRE	Interferon-sensitive response elements
JAK	Janus-kinase
LFTs	Liver Function Tests
MHC	Major histocompatability complex
mRNA	Messenger ribonucleic acid
NANBH	Non- A non-B viral hepatitis
NS	Nonstructural proteins
NCBI.	National Center for Biotechnology Information
OAS	2',5'-oligoadenylate synthetase
PKR	RNA-dependent protein kinase
PVD	Portal Vein Diameter
Peg	Pegylated
PRRs	pattern-recognition receptors ()
PDCs	Plasmacytoid DCs
PKR	Pak rupees
PT	Prothrombin time
PTTK	Partial Thormboplastin Time with Kaolin
RT	Room Temperature
RNA	Rribonucleic acid
SD	Standard deviation
SES	Socio-economic status
STAT1	Signal Transducer and Activator of Transcription 1
TH1 cells	T-Helper cells 1
TH2 cells	T-Helper cells 2
TM	Melting Temperature

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Thesis Outline

Background: Hepatitis C virus (HCV) is a leading cause of hepatocellular carcinoma (HCC) and cirrhosis. More than 3% of the world population and about 6% of Pakistanis are currently living with the virus (genotype 3 most prevalent). Interferon alpha (IFN-α) plus ribavirin is widely used standard therapy for chronic HCV treatment all over the world but pegylated interferon along with ribavirin has higher response rate than that of standard interferon.

Objective of the study: In this study focus has been on to evaluate IFN receptor subunit expression as a predictive factor for response to IFN therapy and disease severity in the population of Pakistan. The other objectives were to analyze the socioeconomic status of the infected individuals and their awareness level regarding risks factors of HCV prevalence in this region.

Principal findings: It is found that forty six percent of the IFN resistant HCV infected patients expressed IFNAR1 mRNA. IFNAR2 and STAT1 were expressed by almost all patients. However, a significant difference is observed in age, BMI, liver enzyme levels, ultrasonic findings and liver histopathology of the patients with positive liver expression of IFNAR1 as compared to patients negative for IFNAR1 expression. HCV infected patients belong to low socio-economic strata of Pakistani population. It is also evident through our statistical data that most of the patients knew that main spreading cause is blood products as compared to other risk factors.

Conclusion: It is contended that IFNAR1 can be used as a predictor factor of response to IFN standard therapy as well as disease severity. Measures should be taken to educate people regarding possible risks of acquiring HCV on urgent basis. Policies should be approved at governmental level for timely diagnosis and prevention of this devastating virus. Socio-economic status should be considered another risk factor for HCV infection and future studies should target the low socio-economic groups for research and other policies making purposes.

Chapter 1

INTRODUCTION

1. Hepatitis C Virus

a. Discovery of HCV

From 1982 to 1988, ~6 years of exhaustive investigations at Chiron Corporation led to the discovery of hepatitis C virus (HCV), flavi-like virus. Subsequently, the research demonstrated that major cause behind parenteral transmission of non- A non-B viral hepatitis abbreviated as, NANBH was HCV around the globe infecting ~170 million people (Houghton, 2009)

b. Classification

The viruses have been classified on the basis of physical as well as biological criteria. David Baltimore devised a classification scheme of the viruses, more than 30 years ago, based on the nucleic acid nature of the virus. The Baltimore classification system, a well-designed molecular algorithm for virologists, encompasses 6 classes of viral genome. The principles in the classification scheme help understand flow of genetic information with different configurations of the viruses (Racaniello, 2009).

Group:

HCV belongs to class IV i.e., positive sensed single-stranded RNA viruses. Positive sense RNA can be directly translated to form proteins by host ribosomes (Nicolini et al., 2012)

Family and Genus:

HCV belongs to the family *Flaviviridae (*large group of viruses) comprising 3 genera i.e., genus *Flavivirus* (e.g., yellow fever virus), genus *Hepacivirus* (e.g., hepatitis C virus), and genus *Pestivirus* (e.g., bovine virus diarrhea) (Beth et al., 2009).

c. HCV: Structure

Size of HCV particles ranges between 40 and 70 nm in diameter with core and envelope glycoproteins i.e., E1 and E2 the chief components. Presumably the envelop proteins i.e., E1 and E2 are anchored in lipid bilayer derived from the host cell which surrounds the nucleocapsid. Nucleocapsid is made of core protein (multiple copies) harboring the genomic RNA (Moradpour et al., 2007).

d. HCV: Genome

The HCV has single-stranded positive sensed RNA genome ~10kb size. The RNA contains an open reading frame and 5' and 3' flanking regions contain untranslated regions (UTRs) as shown in the figure. The open reading frame contains information for viral protein, RNA synthesis as well as for coordination of both processes (Rehermann and Nascimbeni, 2005).

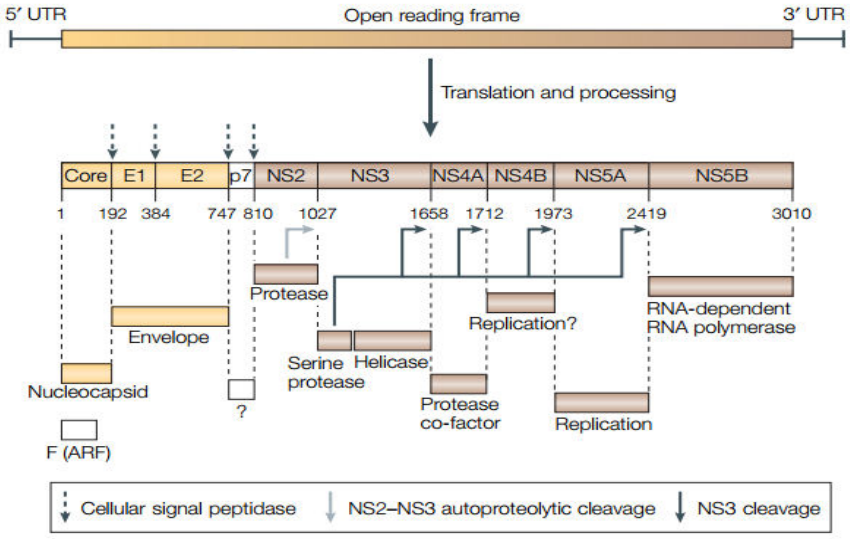


Fig 1: HCV Genome Structure (Taken from Rehermann et al., 2005) (Rehermann and Nascimbeni, 2005)

In the host cell cytoplasm, HCV genome acts as mRNA and is directly translated through 5' UTR Internal Ribosomal Entry Site (IRES). The HCV RNA does not enter the host cell nucleus. The open reading frame is translated as polyprotein which is then processed co- and post-translationally with the help of both cellular as well as viral proteases as follows:

- Structural proteins
    - Core, envelope proteins(E1 and E2) and p7
- Nonstructural proteins
    - NS2, NS3, NS4A, NS4B, NS5A, NS5B (Rehermann and Nascimbeni, 2005)

e. Replication

Following protein synthesis and maturation, membrane-associated replication complex is formed by non-structural proteins and HCVRNA giving an appearance of a perinuclear membranous web. The replication complexes-mediated transcription of negative sensed RNA intermediates in turn generate progeny of positive sensed RNA. Genomic RNA along with capsid proteins assemble resulting in the formation of nucleocapsid which bud through intracellular membranes (Rehermann and Nascimbeni, 2005).

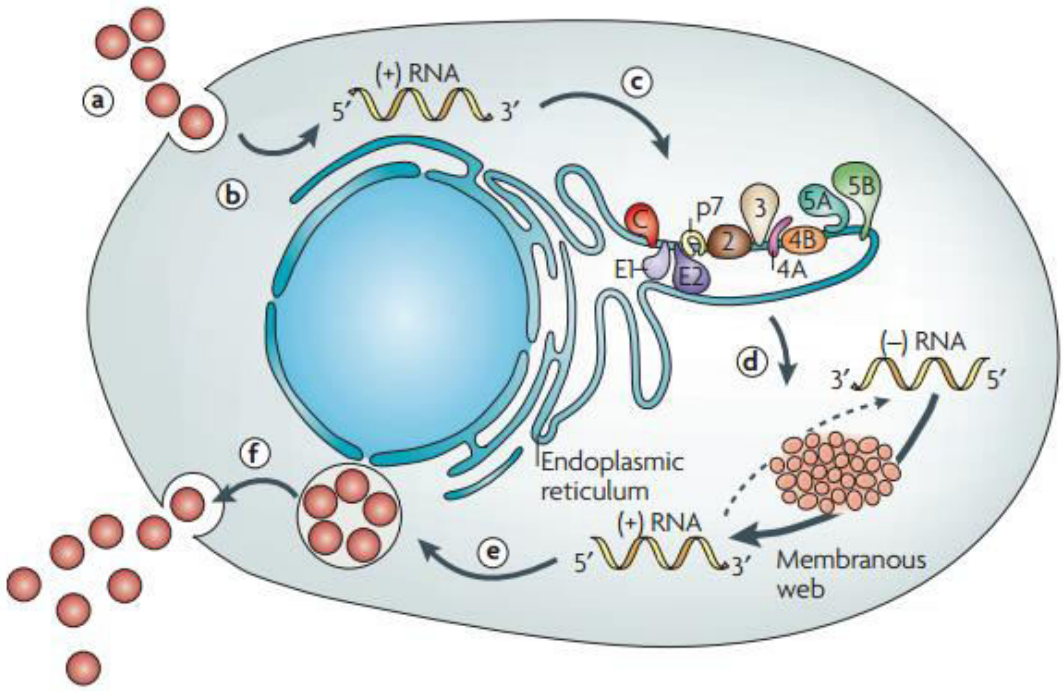


Fig 2 HCV: Life Cycle (Taken from Moradpour et al., 2007) (Moradpour et al., 2007)

f. Genotypes

HCV has high genetic variability. There are many classification systems used for HCV but the one by Simmonds is widely accepted that categorizes HCV in to 11 genotypes with 80 subtypes (Alexopoulou, 2001). The HCV RNA-dependent RNA polymerase lacks proofreading activity which results in greater genetic variability as shown in the figure (Argentini et al., 2009).

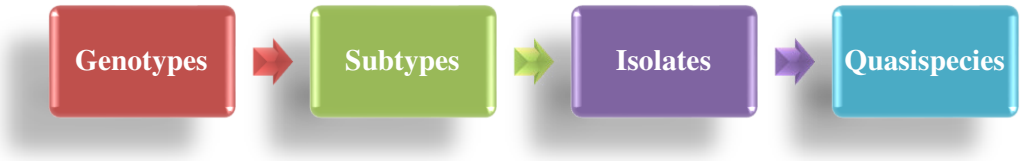


Fig 3: HCV Genetic variability

Genotyping and serotyping techniques were used for HCV genotype determination. The genetic variability of HCV is significantly related to disease severity, prognosis, diagnosis, treatment response and it is the root cause behind failed efforts for HCV vaccine production. Low response to IFN-α/ IFN-α along with ribavirin has been associated with Genotype 1 and 4 infected HCV patients (Alexopoulou, 2001).

g. Vaccination

Different factors were behind failure to develop vaccines against HCV

- Research tools availability
- HCV variability

- Tissue tropism
- Host selection

Establishment of tissue culture system for HCV has enabled the generation of HCV in sufficient amounts for vaccine antigens and immunological bioassay. Nowadays, a number of vaccines have gone into animal as well as human clinical trials.

The main objectives of therapeutic vaccines are;

- Cytotoxic cell activation
- Cross-genotype neutralizing antibodies production

Due to HCV genetic variability, vaccination, anti-HCV treatment or immune modulation can be used in combination. Most of the vaccines are in preclinical stage, some are in phase I/ II trials (Yu and Chiang, 2010).

2. Hepatitis C

The clinical features of hepatitis C are summarized in table 1:

a. Epidemiology worldwide

It has been estimated that ~3% i.e., 170 million of world's population is victim of HCV. Most of the countries have HCV infection prevalence less than 3%, some countries of Africa and Asia have even higher prevalence as shown in figure 4 (Holmberg, 2012).

b. HCV Genotypes Epidemiology

The geographical distribution of HCV genotypes is distinctive globally as shown in the table 2 (Negro and Alberti, 2011).

Table 1. Hepatitis C: Clinical features (Taken from (Rehermann and Nascimbeni, 2005))

Feature	Hepatitis C
Public-health impact	
Worldwide	170 million people infected
Clinical course of infection	
Vertical/perinatal transmission	Rare
Horizontal transmission	Intravenous drug use, parenteral, sexual
Chronic hepatitis: Histological characteristics	Lymphoid aggregation and organization same like primary lymphoid follicles; steatosis (with HCV genotype 3); reactive changes in epithelium of bile ducts
Disease progression	
Liver cirrhosis	5–10% after 10 years of infection
Hepatocellular carcinoma (HCC)	In western world, HCC incidence (5-year cumulative) in cirrhosis patients is 17%, in Japan the incidence is 30%; In Europe and USA , 3.7 per 100 person years in cirrhosis patients ; in Japan it is 7.1 per 100 person years in cirrhosis patients
Preventive vaccination	No
Therapy	Pegylated interferon-α in combination with ribavirin; treatment response in 45–80% of cases, HCV genotype major factor

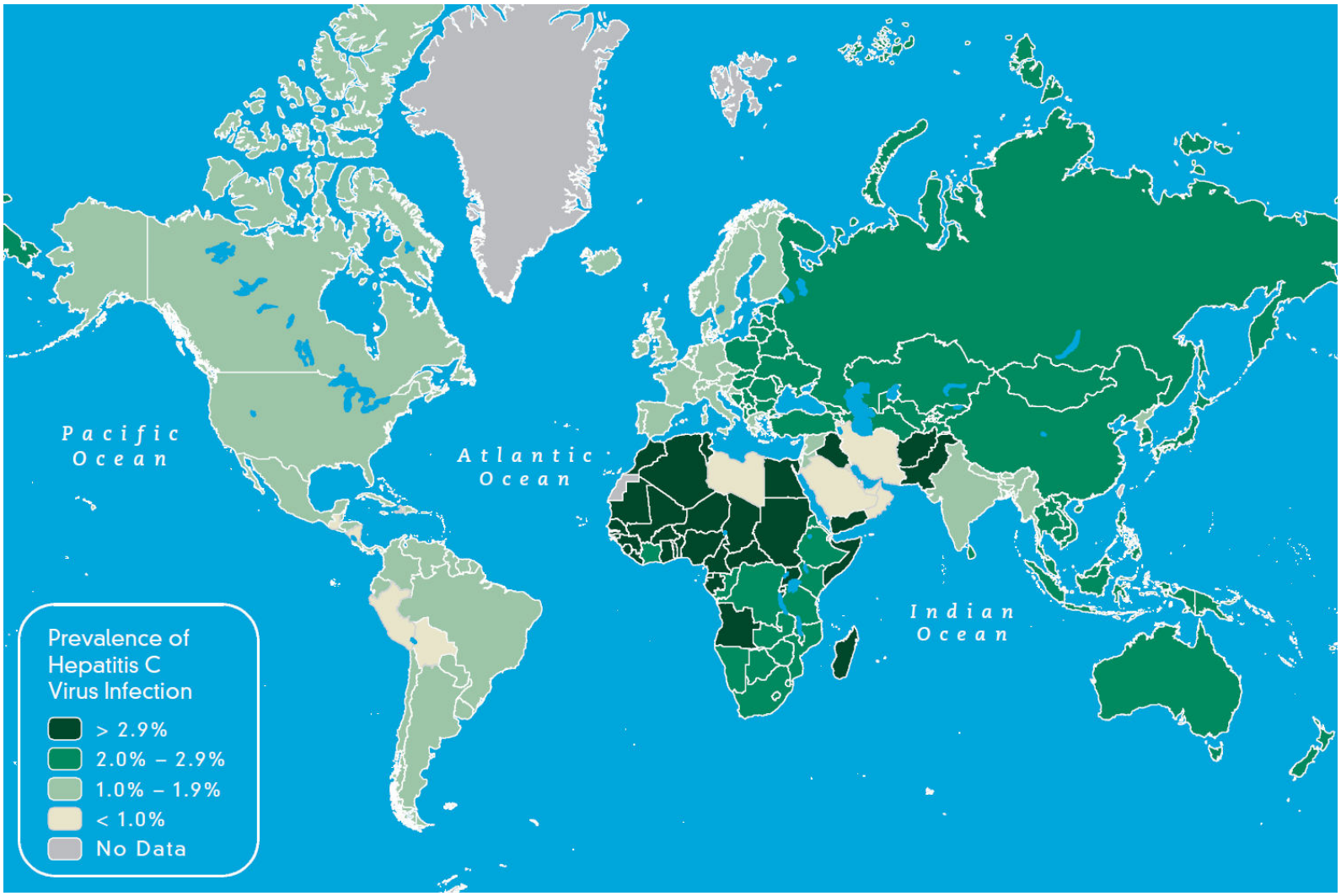
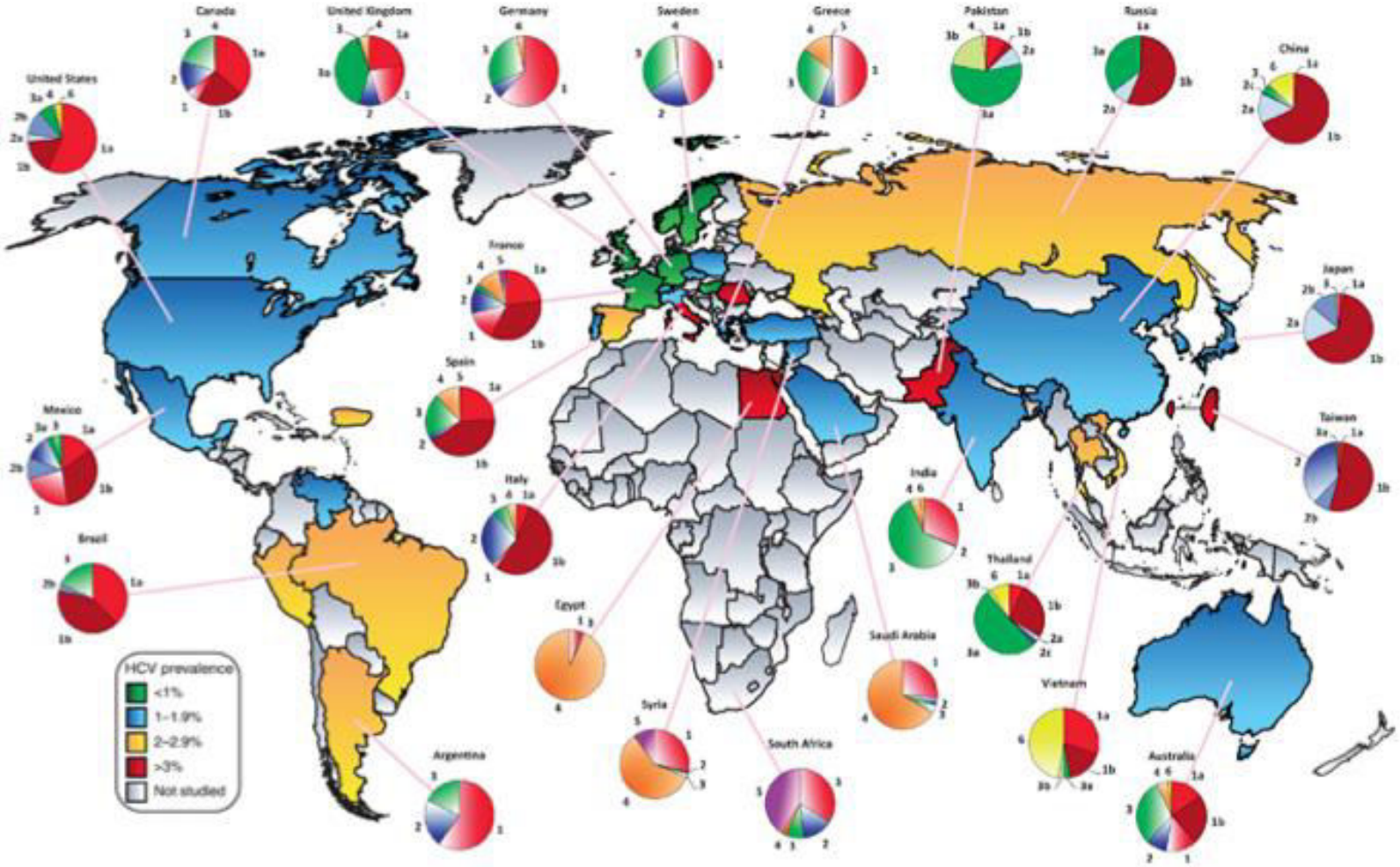


Fig 4: Hepatitis C: Worldwide Epidemiology (Taken from (Holmberg, 2012))

Table 2: Prevalence of HCV and distribution of genotypes (Taken from (Negro and Alberti, 2011))



c. Epidemiology in Pakistan

Pakistan has 6% HCV prevalence in its population (Khan et al., 2013) with genotype 3 predominant (Attaullah et al., 2011). The top three risk factors associated with HCV in Pakistan are Syringe/needle reuse (61.45%), Surgery/dental work (10.62%) and Blood transfusion (4.26%) (Sievert et al., 2011).

In Pakistan, HCV genotype 1 has 7.03% prevalence, genotype 2 has 3.81%, genotype 3 has 78.96%, genotype 4 has 1.59%, genotype 5 has 0.10%, genotype 6 has 0.13%, mixed genotypes have 5.03% and untypeable genotypes have 3.30% prevalence. The predominant genotype is 3a with 55.10% prevalence rate followed by genotype 1a (10.25%), 3b (8.20%) and mixed genotype (5.08%) with respect to prevalence rate whereas genotypes 4, 5 and 6 were not frequent. Genotype 3 is predominates in all provinces. In Punjab province, genotype 1 is second most frequent genotype whereas in provinces Sindh, Balochistan and Khyber Pakhtunkhwa, the untypeable genotypes are the second most frequent genotypes (Attaullah et al., 2011).

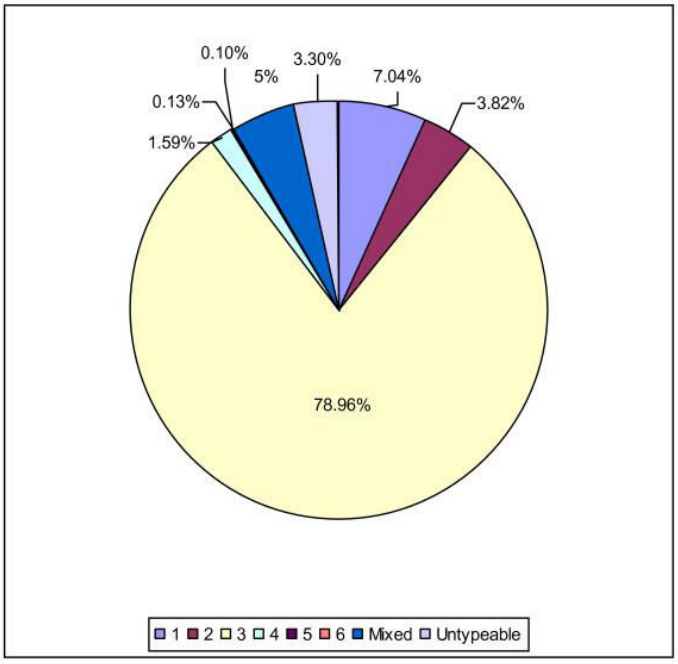


Fig 5: HCV Genotypes Distribution in Pakistan (Taken from (Attaullah et al., 2011))

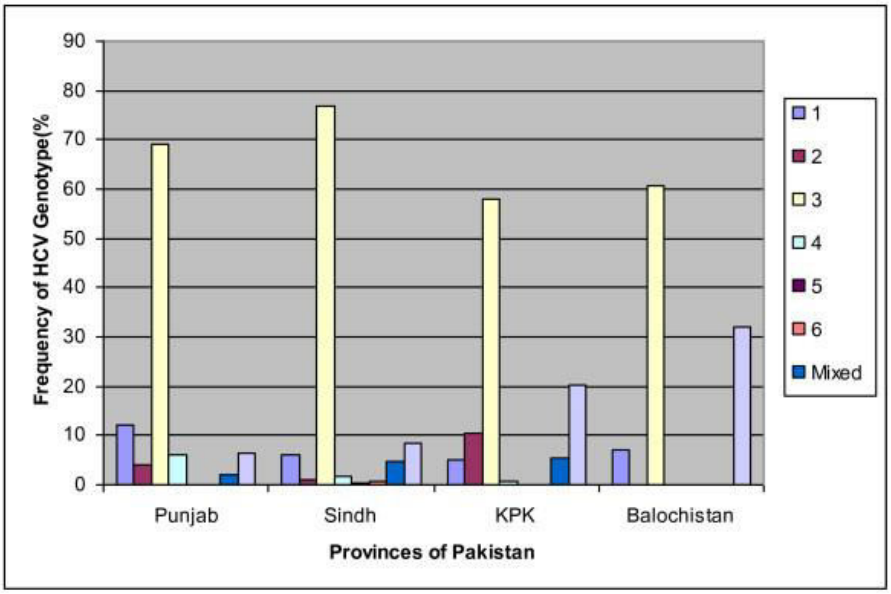


Fig 6: Province Wise HCV Genotypes Distribution in Pakistan (Taken from (Attaullah et al., 2011))

d. Signs and symptoms

Most of the HCV infected patients i.e., 60%–75% show no symptoms. In case of symptomatic acute infections, the symptoms include jaundice, fatigue, malaise, anorexia, lethargy, abdominal pain, arthralgia, mild hepatosplenomegaly and maculo-papular rash. These symptoms may persist for 2–12 weeks. Fulminant hepatitis is very uncommon in acute infection stage (Wong and Lee, 2006).

e. How Hepatitis C spreads?

Blood products are the main source of transmission of Hepatitis C. Besides this, sharing contaminated needles (e.g., injection drug users), reuse of contaminated syringes/ medical equipment, hemodialysis, surgery and infected sex partner can spread HCV infection (Wilkins et al., 2010). In Punjabi population the risk factors for HCV transmission include therapeutic injections (44.3%), dental surgery (16.6%), Blood contact or transfusion (10.7%), shaving at barbers shop/ sharing blades (5.9%), surgeries/ medical procedures (3.3%), multiple risk factors (9%), sporadic (4.9%) and others (5.4%) (Mujtaba et al., 2011). Mother to child transmission ranges from 2-8% (Prasad and Honegger, 2013). No evidence was found about association of casual contacts or sharing utensils with transmission of HCV (Lo Re and Kostman, 2005). Sneezing and coughing are also not the sources of HCV transmission (Arend, 2000). Oral fluid mode of HCV transmission is a controversy (Suzuki et al., 2005) but Saliva has been found positive for HCV in many studies (Arend, 2000, Suzuki et al., 2005).

f. Treatment

IFN-α along with ribavirin is currently a standard treatment for HCV but the therapy is successful in ~50% of the patients with adverse affects. Through next decade, the pegylated IFN-α will stay base for anti-HCV treatment because of the fact that new formulations of IFN are not superior to this one or are in early developmental stages. Partial antiviral activity has been demonstrated by antivirals that act directly and hence are not adequate to be used as monotherapy (Vezali et al., 2011).

The combination of Peg-IFN-α2b/ Peg-IFN-α2a with ribavirin which has successful therapeutic response in 40–50% of HCV genotype 1 or 4 infected patients and 75–90% in HCV genotype 2 or 3 infected patients. The side effects commonly associated with this therapy includes reduced life quality resulting in dose reduction/ discontinuation in some patients (Sulkowski et al., 2011).

As the HCV treatment has difficulties both physically and economically with failure rate in about 50% of the patients, this marks the importance of understanding the underlying mechanism in interferon resistance or non-response to therapy. Identification of factors behind non-responsiveness can be helpful in response prediction for the treatment. Resistance to therapy is the result of different factors including viral factors, host factors and HCV proteins induced molecular mechanism that inhibits IFN signaling cascade (Asselah et al., 2010).

3. Interferon

Isaacs and Lindenmann discovered interferon- α/β (IFN- α/β) in 1957. It is a family of cytokines playing critical role in innate immunity regulation through its pleiotropic actions on almost all types of somatic cells. Humans express about 20 IFN-α subtype genes along with individual genes that encode IFN –β, -κ, -ω and –ε (Huber and Farrar, 2011) . In humans, Interferons (IFNs) are key physiological regulators playing important role in biological functions with excellent pharmacological properties against a number of diseases e.g., viral infections and malignancies (Uddin and Platanias, 2004).

a. Interferons and Interferon Receptors: Classification

There are two major groups of IFNs i.e., type I and type II interferons. Type I interferon include IFNα, IFNβ and IFNω whereas type II interferons include IFNγ. Genes encoding type I IFNs are located on chromosome 9. Structurally, IFNα, IFNβ and IFNω have significant homology with each other and minimal homology with IFNγ. All interferons bind to specific cell surface receptors hence initiating their biological effects. Type I IFNs bind specifically to type I IFN receptor whereas type II IFNs binds to type II IFN receptor. The type I IFN receptor (IFNR) consists of two subunits i.e., IFNAR1 and IFNAR2. IFNAR1 is 110 kDa in molecular mass whereas IFNAR2 has two different forms because of differential splicing of same gene i.e., IFNAR2c form (90-100 kDa) and IFNAR2b form (51 kDa). Type II IFN receptor consists of two subunits i.e., IFNGR1 (90 kDa) and IFNGR2 (62 kDa). Type I IFN

signal transduction mediates multiple biological functions such as Immunomodulation, differentiation, apoptosis, anti-angiogenic effect, cell cycle arrest, anti-viral effects, anti-tumor effect and protein synthesis as shown in Figure (Uddin and Platanias, 2004).

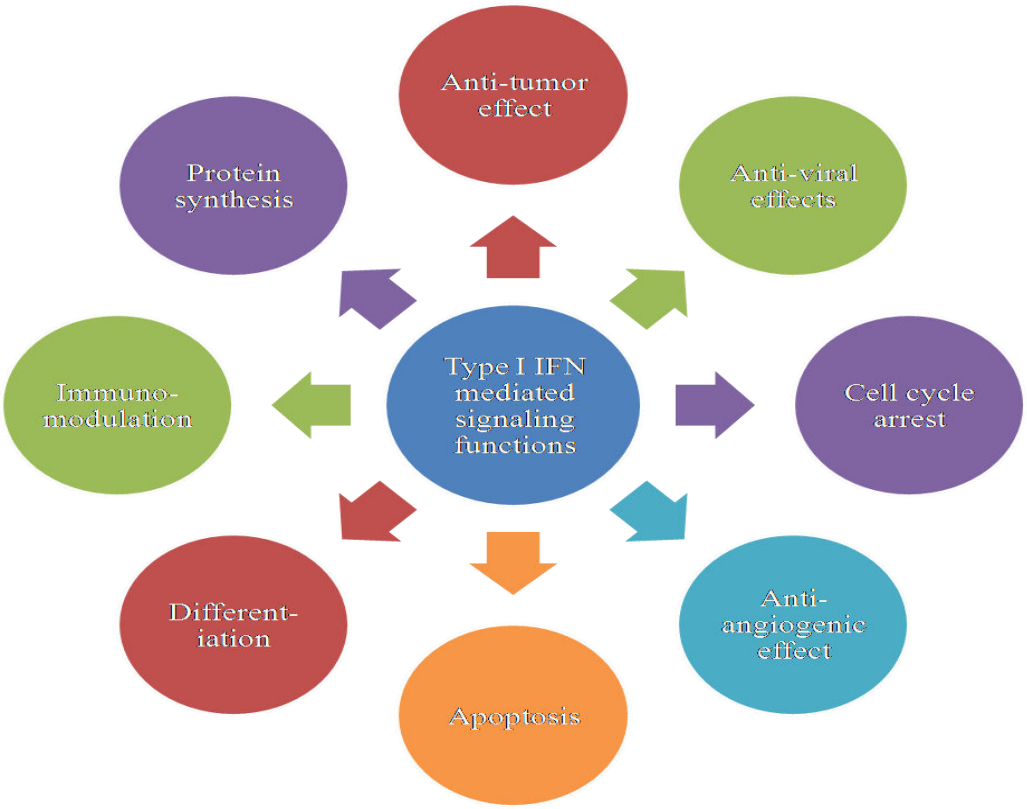


Fig 7. Type I IFN mediates multiple biological functions (from (Uddin and Platanias, 2004)

b. Induction and Regulation of interferon

Cells of the innate immune system i.e., macrophages and dendritic cells (DCs) sense pathogens through their pattern-recognition receptors (PRRs) and produce type I IFNs. Plasmacytoid DCs (pDCs) particularly produce IFNα in large quantities

whereas non-immune cells i.e., epithelial cells and fibroblasts produce IFNβ predominantly. Type I IFNs limit infectious agents spreading through induction of IFN-stimulated genes (ISGs) expression in infected and neighbouring cells. In response to type I IFNs, innate immune cells enhance antigen presentation and produce immune response intermediaries i.e., cytokines and chemokines. B cells produce antibodies and T cells effector function is amplified in response to type I IFNs as part of adaptive immunity (as shown in figure 8). (Ivashkiv and Donlin, 2014).

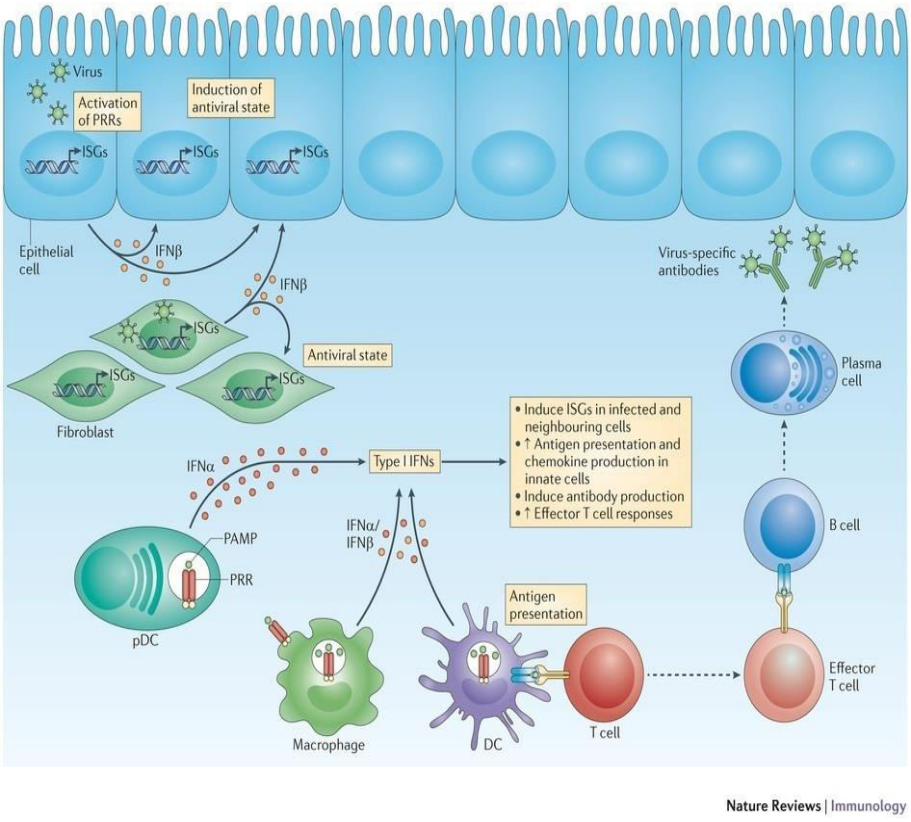


Fig 8: Type I interferon controlling innate as well as adaptive immune response along with antimicrobial programmes with in cell (Taken from Ivashkiv et al., 2014 (Ivashkiv and Donlin, 2014)).

c. Mechanism of IFN Action

IFN- α/β receptor (IFNAR), expressed ubiquitously and constitutively, is a heterodimer consisting of two subunits namely R1 and R2. IFN- α/β interaction with the receptor results in its dimerization and in turn activation of the JAKs which phospohorylate IFNAR1/2 cytolpasmic domains. STAT1 and STAT2 are recruited to the receptor, phosphorylated and then associate with interferon regulatory factor-9 resulting in the formation of interferon-sensitive gene factor-3 (ISGF3). ISGF3 then translocates in the nucleus to regulate interferon-sensitive genes (ISGs) expression by transactivation of the interferon-sensitive response elements (ISRE) (Huber and Farrar, 2011).

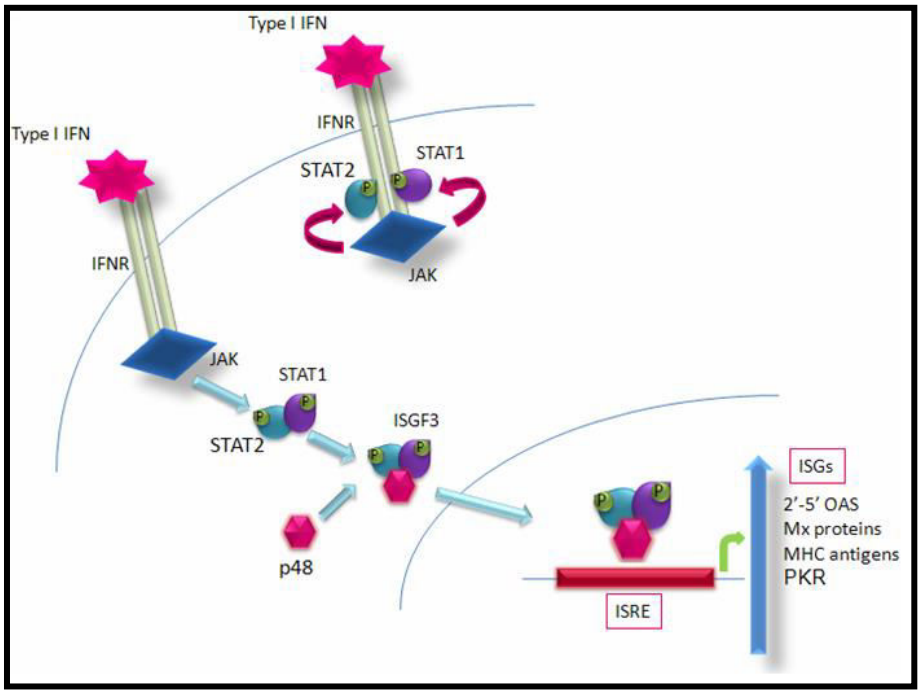


Fig 9: Type I Interferon: Signal Transduction (Figure redrawn from (Taylor et al., 2000))

IFN-inducible genes and their subsequent proteins, that inhibit virus replication, include RNA-dependent protein kinase (PKR), 2',5'-oligoadenylate synthetase (OAS), Major histocompatability complex (MHC) and Mx proteins. PKR plays role by inhibiting translation initiation and 2',5' OAS mediates degradation of RNA. IFN-α triggers MHC class I antigen expression in immune cells and activates effector cells besides interaction with cytokine cascade. IFN- α also performs immune-modulatory functions by stimulating TH1 cells production whereas reducing the production of TH2 cells. The whole mechanism result in antiviral state within infected cells that directly inhibits viral replication at the same time it also enhances antiviral immune responses of the host (Vezali et al., 2011). The JAK-STAT pathway malfunctioning can lead to pathogenesis in some human diseases (Igaz et al., 2001).

4. Molecular Mechanism responsible for Interferon Resistance

To date IFN and ribavirin are the only successful HCV treatment regimen availabe. HCV evolved diverse mechanisms to evade IFN response through blocking downstream signaling i.e., STAT1, STAT2, IRF9 and JAK-STAT pathways and IFN Stimulatory Genes (ISGs) repertoire (Qashqari et al., 2013). According to a number of studies, host factors (demographic and genetic) and viral factors i.e., genetic heterogeneity affects IFN therapy outcome (Qashqari et al., 2013, Asselah et al., 2010). Many studies demonstrated up-regulation of some ISGs in non-responders before treatment. Clinical, biochemical and histological findings can predict the therapy response before treatment starts. This is of great concern because standard treatment for HCV is physically as well as economically demanding. Different factors

are associated with IFN therapy failure i.e., viral factors, host factors and HCV induced molecular mechanisms that inhibit IFN signaling pathway. In order to determine treatment response, gene expression analysis has been done in liver biopsies. (Asselah et al., 2010). Among the liver genes, the decreased expression of IFN-α receptor might be the cause of resistance to therapy in HCV patients. Responders have shown higher levels of receptor mRNAs in liver compared to non-responders (Ahmad et al., 2012). A study showed 97% of HCV patients expressing IFN-α mRNA whereas 30% of HCV patients expressing IFN-α receptor mRNA and hence concluded that lower IFN-α receptor expression is associated partially with interferon resistance to therapy in these patients (Salama H, 2007). As the expression of IFNAR1 and IFNAR2 is key to success of IFN- α treatment in viral hepatitis hence the receptor expression downregulation could result in treatment failure. So treatment success depends largely on levels of IFN- α receptor expression and can be a predictor of response to therapy. Fibrotic livers also have downregulated expression of the IFN-α receptor (Gao et al., 2004). IFN-resistant phenotypes (partially IFN resistant & highly IFN resistant) possessing HCV replicon-harbouring cell lines when used to evaluate viral and cellular factors responsible for IFN resistance, results showed that cellular factors contributed to IFN-resistance including observation that genetic abnormality in IFN-α receptor also resulted in interferon resistance (Naka et al., 2005a).

Identifying the factors responsible for non-responsiveness can be helpful in predicting the treatment response. Resistance to therapy can be result of different

factors including viral factors, host factors and HCV proteins induced molecular mechanism that inhibits IFN signaling cascade (Asselah et al., 2010). It has been observed that cellular factors as compared to viral factors contribute to a phenotype that is highly resistant to IFN (Naka et al., 2005b). Though the world has progressed in different aspects to figure out the main defects associated with the interferon resistance in HCV patients, in Pakistan, the role of interferon receptors in HCV patients resistant to therapy has not been investigated. Neither the molecular nor immunological level studies have taken into consideration to figure out this pivotal/ critical issue in Pakistan. So keeping in view the importance of the cellular factors, a study was designed based on analyzing cellular factors that are thought to be key to success for the therapy i.e., Interferon alpha receptor. The current study was aimed to investigate the qualitative expression analysis of IFN-α receptor among HCV infected patients resistant to standard interferon therapy and disease severity. Along with this, the risk factors exposed to subjects included in the study along with their awareness level regarding HCV was also evaluated.

5. Risk Factors and Socioeconomic Status of HCV Infected Patients

Risk factors regarding HCV infection vary significantly among countries and geographic regions (Karaca et al., 2006). Pakistan is one of the nations that are worst affected by HCV and mortality as result of liver failure and HCC. Taking into consideration the size of Pakistan and its large growing population, there are no estimates available at national level regarding prevalence of hepatitis and associated risk factors. Much is known about risk factors as evident from the published literature

in Pakistan (Ali et al., 2009). We also identified the possible risk factors among the population that we selected for this research.

Hepatitis C causes many extrahepatic manifestations ultimately resulting in poor health-related quality of life (HRQOL). There are many other factors behind poor quality of life in HCV patients including physical as well as psychological factors (Abdo, 2008) including change in behavior, internalized shame, financial insecurity and social rejection (Zacks et al., 2006).

Socioeconomic status (SES) is the position of a person in a society and can be estimated by criteria like education, job/occupation and income. Studies from USA, Norway, France and Puerto Rico are evident for associations between HCV prevalence and different markers of SES. An increase in risk of acquiring HCV infection has been observed in people with low SES (Omland et al., 2013). The current study also aimed to investigate the possible involvement of SES as risk factor for HCV infection.

6. Basic HCV Awareness

Many persons are not aware of HCV infection because of asymptomatic and hence they do not undergo for HCV screening unless they develop complications of liver and then seek for medical care which is also a small portion of people among the infected patients who are unaware that they have acquired HCV infection (Denniston et al., 2012). Hence awareness is very important to manage and treat HCV. Recent studies show that awareness level regarding HCV is extremely low among general Pakistani population especially among women and people who are less educated

(Jamil et al., 2010, Asif et al., 2009). Healthcare workers are at higher risk of HCV infection. Studies have shown that most of the sharps injuries were sustained while drawing blood or injecting practices. Wearing gloves can help avoid infection contraction. Medical students are at higher risk of acquiring infections as they deal patients, have direct contact with them and with blood (transfusions) and surgical instruments/injections. There is a need to start training regarding infectious diseases precautions in countries lacking safety equipment, safety instructions for infectious diseases and staff vaccination programmes (Anjum et al., 2005). Most of HCV/ HIV patients visit hospital daily for consultations, admissions, procedures and diagnosis and hence encounter health care workers. Medical students (also included in healthcare delivery system) are at high risk as they also deal with patients and infected instruments (Saleem et al., 2010). Usually the medical students are associated with the activities related to care of the patient and are first level of contacts with the patients in start of their clinical years. Being future doctors, at the start of the clinical rotations, they are at high risk of infections because of lack of experience and skill. At this stage they must have sound knowledge of the blood borne diseases like HCV infection as medical student's awareness for HCV is very important not only for their own safety but also for the effective patient care with whom they are in direct contact. Misconceptions regarding basic knowledge for HCV may put them at higher risk as well as can hinder the effective interaction between patient and doctor. Lack of knowledge regarding actual routes of HCV transmission can put the student at high risk of getting HCV infection and on the other side, misconception regarding HCV

transmission route can hinder the student to keep in close contact with the patient, take care of the subject and his/her better treatment.

Objective of the Study:

- The main objective of the study was to analyze the expression of IFNAR1, IFNAR2 and STAT1 in HCV infected patients resistant to standard interferon therapy by qualitative PCR analysis and their relation with clinical investigations e.g., liver function tests, viral load, liver histopathology i.e., disease severity in Pakistani subjects.
- The second main objective of the study was to estimate the basic awareness level about HCV transmission routes and risk factors among normal healthy subjects, subjects with history of HCV infection and medical students who are at the start of their clinical rotation.
- The third main objective was to find out the major risk factors exposed to subjects included in the study and evaluation of socioeconomic status of hepatitis C infected patients as a possible risk factor keeping in view the high cost of HCV diagnosis and treatment (economic burden on common person and HCV burden in the country).

Chapter 2

MATERIALS AND METHODOLOGY

2.1 ETHICAL COMMITTEE APPROVAL

The study was conducted after formal approval was obtained from the Ethics Committee, Army Medical College, Rawalpindi. Informed written consent was obtained from all the subjects enrolled in the study. Those who refused to take part in the study were not included in the final data analysis.

2.2 STUDY DESIGN

The study consisted of qualitative as well as quantitative methodologies to understand the reasons why HCV risk and prevalence in our population is getting higher day by day and why this virus is not responding to interferon treatment.

Defining the sample population for qualitative research

For qualitative research we conducted comprised structured, self-administered questionnaire based cross sectional study that included the following groups:

1. Subjects having HCV infection history including responders (N=15) as well as non-responders to interferon therapy (N=50)
2. Normal healthy subjects (N=15). One lady didn't participate in the study so she was not included in the final data analysis.

3. For awareness survey, 179 medical students were also included in the studies that are at the start of clinical rotation.

   The subjects included in the study were selected based on convenience sampling method.

The subjects included in the qualitative research were assessed for

1. Awareness regarding routes of HCV transmission
2. Risk factor exposure during their life time that can be possible sources of acquiring HCV infection.
3. Socioeconomic status (SES) of the subjects included in the study. Because SES is thought to be an emerging and most dangerous risk factor for HCV transmission.
4. The total cost of HCV diagnosis and treatment was also estimated to explain the reason why people are not getting timely diagnosis and follow complete treatment plan for this asymptomatic disease. This is one of the very important reasons for high burden on the finance of the common man and high prevalence of HCV in Pakistan.

Defining the sample population for quantitative research

For quantitative research, the main focus was on HCV infected patients resistant to standard interferon therapy included in the study based on convenience sampling method. The following groups were also included in the group along with the above mentioned group:

Group I: A control group of normal healthy individuals (n=15) both males and females i.e., those negative for HCV, other viral hepatitis and HIV.

Group II: Second control group comprised of individuals (n=15) both males and females that were previously infected with HCV but responded to standard interferon treatment (i.e., they received subcutaneous injections of recombinant IFN-α with 3 million IU dose thrice weekly and ribavirin with 10 mg/ kg body weight/ day with 24 weeks therapy duration) and were negative for serum HCV RNA, other viral hepatitis and HIV.

Group III: Individuals infected with HCV that didn't respond to twice course of standard interferon treatment (n=50). Both male and female patients were included in the study. The other inclusion criteria were patients positive for anti-HCV antibodies and serum HCV RNA with or without elevated ALT levels. HCV genotype 3 patients were included in the study. The exclusion criteria for the study were HCV patients co-infected with other viral hepatitis or HIV, abnormal bleeding time, platelet count and prothrombin time.

The subjects included in the quantitative research were assessed for

1. Demographic features
2. Body mass index
3. Blood tests
    a. Pre-biopsy tests
        i. Bleeding time

ii. Clotting time

iii. Prothrombin time (PT)/Partial thormboplastin time with kaolin (PTTK)

The patients normal for pre-biopsy tests were then undergone for liver biopsy.

b. Liver function tests

i. Alanine Aminotransferase (ALT) estimation

ii. Alkaline Phosphatase (ALP) estimation

iii. Bilirubin estimation

c. ELISA based screening of viral hepatitis and HIV/ AIDS

d. Viral load estimation

e. HCV genotyping (HCV genotype 3 infected patients were included in the study)

4. Abdominal ultrasound
5. Liver biopsy and histopathology
6. PCR based expression detection
    a. IFNAR1
    b. IFNAR2
    c. STAT1

2.3 QUESTIONNAIRE

The questionnaire (Appendix I) was designed and assessed for its validity by a panel of clinical experts. The questionnaire had four parts. First part was for demographic data comprising of participant's age, gender and marital state. Second part was focused on knowledge of risk factors associated with HCV infection and comprised of questions to evaluate the knowledge level of the participants about routes of HCV infection and risk factors associated. Third part was to evaluate socio-economic status focusing on three markers i.e., education, employment/occupation and income. Forth part was focused on probable exposure to risk factors that can be important for acquiring HCV comprising detailed interview of the patients. The participants of the study were briefed about the purpose of the study and assured about the data confidentiality. Filling a questionnaire took around 20 minutes by each participant. The data was coded and then analyzed using SPSS for Windows version 20.0. Awareness questionnaire was adopted from (Mayor et al., 2010) and (Tiftikci et al., 2009) with some modifications, the questionnaire is attached as annexure.. Risk factors exposure questionnaire was adopted from the articles by Ghias (Ghias and Pervaiz, 2009, Ghias et al., 2010) with some modifications. Three markers of socioeconomic status as mentioned by Omland (Omland et al., 2013) were also determined. We also approximately estimated the cost of diagnostic tests and treatment for HCV in Pakistan.

2.4 SAMPLE COLLECTION

Blood as well as biopsy samples were taken from HCV infected patients resistant and responder to standard interferon therapy reporting to the department of medicine, Military Hospital, Rawalpindi. Biopsy samples were not available for normal healthy individuals and previously HCV infected subjects who responded to IFN treatment hence the blood was used for further analysis. Blood was processed the same day for RNA extraction and liver biopsies were stored at -80°C for further processing.

A. Blood Sample Collection

Blood samples were collected from all participants of the quantitative research under fully aseptic conditions. All specimen tubes were marked with the identification of the patient. Blood was drawn from the median cubital vein. The tourniquet was placed on the upper part of the arm (3 to 4 inches above the venipuncture site), tight enough to make the vein bulge. The sample was collected in the specimen tubes which were properly labeled with patient's particulars. The needle was disposed off by using the cutters. The samples were then transported in liquid nitrogen from MH (Military Hospital, Rawalpindi)/ CMH (Combined Military Hospital, Rawalpindi) to CREAM (Center for Research in Experimental & Applied Medicine), department of Biochemistry, Army Medical College, Rawalpindi. The sample was processed for RNA extraction the same day it was collected.

B. Ultrasound Guided Liver Biopsy

Ultrasound guided liver biopsy was performed to obtain liver sample from the patients. Before arriving for biopsy, patients were asked not to take blood thinners, aspirin/ aspirin-related medications for 7 days before biopsy, not to eat/ drink for 8 hours before biopsy and to take prescribed medications with a little amount of water. Some blood tests were performed before liver biopsy i.e., bleeding time, platelet count, prothrombin time (PT). After arriving for biopsy, brief clinical assessment was done i.e., pulse and blood pressure were measured. Biopsy procedure was explained to the patient. Risks and benefits of biopsy were explained and informed written consent was taken on a consent form indicating willingness of the patient to undergo biopsy procedure and participate in the study. During biopsy, the patient's ultrasound images of abdomen were obtained in order to locate biopsy area precisely. After locating the liver biopsy area, the abdomen was cleaned and sterilized/ disinfected. Sterile gloves were used for the biopsy procedure. For biopsy, special needle device was inserted 3 cm and liver sample was removed. A staple gun like noise was heard marking that sample is taken. After biopsy, patient was observed for four hours to monitor any complications associated with liver biopsy. The patient suffered some pain but he/ she was given the painkiller medications for his/her comfort.

Transportation of specimens

Transportation of the biopsy sample was carried out under strict aseptic conditions. Hands were washed and sanitized before and after sample collection. Samples were placed aseptically in sterile containers. The container was closed tightly during transportation in order to avoid any leakage. The container was labeled and date marked. The specimens were immediately transported to the laboratory for storage after biopsy.

2.5 CLINICAL PARAMTERS

Patients were monitored using following parameters; body mass index (BMI), pre-biopsy tests (Bleeding time, Platelet count, Prothrombin time), histopathological state of the liver (H & E staining), viral load by quantitative PCR, genotype of the HCV, LFTs (ALT, ALP, Bilirubin) and screening for all viral hepatitis.

A. BODY MASS INDEX (BMI)

BMI was calculated using the English system. The BMI was measured by first taking the weight of the patient in pounds (lbs) and height in inches.

Using English system, the BMI formula is as follows:

Weight (pounds: lbs) divided by height (inches (in)) squared and multiplied by 703 (conversion factor to express BMI in SI units i.e., kg/m^2).

$$BMI = \frac{\text{Weight (lbs)}}{\text{Height (in)}^2} \times 703$$

Results interpretation:

For adults i.e., people aged 20 years old and above, the BMI results can be interpreted using the categories of standard weight status (that are same for male and female of all ages). The standard weight status categories and respective BMI values (adults) are as follows:

BMI	Weight status
Below 18.5	Underweight
18.5 to 24.9	Normal
25.0 to 29.9	Overweight
30.0 and above	Obese

B. PRE-BIOPSY TESTS

a. Bleeding Time:

Bleeding time was measured using the Ivy's method.

Requirements:

- Sphygmomanometer
- Lancet
- Circular filter paper
- Stop watch

Procedure:

1. The sphygmomanometer cuff was applied to the patient's arm who was lying supine on a couch.
2. The cuff was inflated to 40mm Hg which was maintained during the test.
3. Forearm volar surface was cleaned with spirit swab and area was chosen without any visible veins.
4. Two cuts 4-8mm long and 1 mm deep separate punctures 5-10cm apart were made along the long axis of the forearm using standard depth lancet.
5. The blood was allowed to flow out freely and stop watch was started.
6. The oozing blood was blotted using the edge of the filter paper (circular) by gentle touch after every 15 sec. The blood was keep on blotting until the blood flow stopped and no more blood spot was seen on blotting paper.
7. The stop watch was stopped and time was noted. This was the bleeding time.

   References values: Ivy's method using lancet: 2-7 minutes

b. CLOTTING TIME:

- Disposable syringes
- Glass test tubes (75x12mm with 10mm bore)
- Water bath for incubation at 37ºC
- Stop watches: 3

Procedure:

1. Three glass test tubes were placed in water bath maintained at temperature 37ºC.

2. The vein puncture site was cleaned with spirit swab and allowed to dry.
3. Then 3mL of blood was collected using sterile disposable syringe. The all of the three stop watches were started as the blood entered the syringe.
4. Then 1mL blood was put in each of 3 glass test tubes placed at 37ºC in water bath.
5. To see the blood clotting, the tubes were tilted initially after 4 min and then every 30 sec.
6. The stop watch was stopped for the tube in which blood clotted. Time was noted for each tube. Mean was taken for three readings. That was the clotting time.

   Reference values: 5-11 minutes

c. PROTHROMBIN TIME (PT)

Requirement:

- Patient's platelet poor plasma: 9:1 ratio of patient blood and trisodium citrate (31.3g/L trisodium dihydrate) was added in plastic tube. Centrifugation was done at 2,000xg for 15 min at 4ºC. The platelet poor supernatant plasma was collected into a plastic tube for testing.
- Normal control plasma: Preparation was done by pooling platelet poor plasma from 4-20 normal healthy subjects.
- Thromboplastin: Commercially available Thromboplastin was used.
- Glass tubes (75x12mm)
- Automatic pipettes (100)

- Water bath
- Stop watches
- Table lamp

Procedure:

1. The table lamp was set on water bath the way that tubes were seen against it but eyes were safe from direct light.
2. Four plain glass tubes were placed in water bath set at 37ºC.
3. Then 100µL test plasma was delivered in a test tube and then wait for 2 min.
4. Then 200µL tissue thromboplastin (commercially available) was delivered and the stop watch was started. The contents were mixed and left for 6-8 sec.
5. The tube was examined after 6-8 sec against light shield by tilting to see any clot formation and then after every 1-2 sec by taking the tube briefly out of water.
6. The stop watch was stopped as the visible clot formation occurred in the test tube and time was noted.
7. The procedure was repeated on test plasma. Then the mean of these two recorded times was taken.
8. The test was then repeated using control plasma.

   Reference values: 10-14 sec

d. PARTIAL THORMBOPLASTIN TIME WITH KAOLIN (PTTK)

Requirements:

- Test and control plasma were prepared as for prothrombin time.
- Platelet substitute used was commercially available.
- Kaolin in barbitone buffer (Ph 7.4)

Sodium diethylbarbiturate	11.74g
Hydrochloric acid	430mL
Kaolin	2.15g

- Calcium chloride
- Automatic pipettes (100µL and 200µL volume)
- Test tubes (plastic and glass) 75x12mm
- Stop watches
- Timer
- Table lamp
- Water bath

Procedure:

1. Equal volume of platelet substitute and kaolin suspension were mixed and left in water bath (to warm).
2. The 75x12mm glass tube was placed in water bath.
3. Then 100µL test plasma was delivered in pre-warmed test tube.
4. Then 200µL platelet substitute-kaolin mixture was added to it. The timer was started. Mixing was done at intervals.

5. Incubated for 10 minutes in water bath.
6. Then 100µL calcium chloride was added and stop watch was started. The clot formation was observed at intervals as in prothrombin time estimation procedure. The stop watch was stopped as soon as the fibrin clot appeared. Time was noted.
7. The procedure was repeated on test plasma. Then the mean of these two recorded times was taken.
9. The test was then repeated using normal pooled plasma.

Reference values: 25-43 sec

C. HISTOPATHOLOGICAL STATE OF THE LIVER (H & E STAINING)

The liver biopsy specimens were fixed in 10% formalin and then embedded in paraffin. Dehydration of the specimen was done by incubating the specimens in 80% alcohol for one hour, 95% alcohol for one hour, 100% Alcohol for one hour and then with a second incubation with fresh 100% Alcohol for one hour. The specimen was cleared by incubating in xylene for two hours and the step was repeated with fresh xylene. Infiltration was done using paraffin with TM (melting temperature) 56-58 °C for two hours and the step was repeated once. Filtered paraffin with TM (melting temperature) 56-58 °C was used for the process of embedding. Metallic molds were used to prepare blocks. Molten paraffin wax was filled in each mold and tissue was carefully placed in the Center of mold. Mold was then allowed to cool at room temperature and then shifted to refrigerator. Sectioning was done by placing the block in block holder of rotary microtome. And sections of 4-5 um thickness were made and

floated in water bath at temperature 45 °C and taken separately on two different slides coated with albumin. Creases were removed by clipping the slides horizontally in 70 % alcohol bath. For about 30 minutes, slides were kept in slanting position draining excessive water. Sections were dried on hot plate for ~15–30 minutes at 60 °C. Staining was done using H & E stain which was used for first slide. To demonstrate fibrosis, reticulin stain was used for second slide. Stained sections were mounted using canada balsam and covered with cover slip. Knodell's scoring system was used to evaluate the extent of liver damage.

D. LIVER FUNCTION TESTS:

i. ALT Estimation:

ALT estimation was done using Liquick Cor-ALAT (Cormay, Poland) kit method (cat# 1-312). Serum was separated from the blood sample. Working reagent (1000µl) was prepared by mixing gently 4 parts of 1-ALAT and 1 part of 2-ALAT avoiding foam formation. Working reagent (1000µl) was pipette into the cuvette and brought up to the temperature of determination (25 ºC). Then 100µl of sample was added to the cuvette, mixed and then incubated for 1 minute at 25 ºC temperature. Absorbance at 340nm was taken against water. The readings were repeated exactly after 1, 2 and 3 minutes. Change in mean absorbance per minute (ΔA/min) was calculated.

ALAT activity [U/l]= ΔA/min. X F (F value=1746 at 340nm)

Reference values (25ºC): Serum/ Plasma of women (up to 42 U/l) and men (up to 36 U/l)

ii. ALP Estimation:

ALP estimation was done using Liquick Cor-ALP (Cormay, Poland) kit method (cat# 1-317). Serum was separated from the blood sample. Working reagent (1000µl) was prepared by mixing gently 4 parts of 1-ALP and 1 part of 2-ALP avoiding foam formation. Working reagent (1000µl) was pipette into the cuvette and brought up to the temperature of determination (37 ° C). Then 20µl of sample was added to the cuvette, mixed and then incubated for 1 minute at 37 ° C temperature. Absorbance at 405nm was taken against water. The readings were repeated exactly after 1, 2 and 3 minutes. Change in mean absorbance per minute (ΔA/min) was calculated.

ALP activity [U/l]= ΔA/min. X F (F value=2764 at 405nm)

Reference values: Serum/ Plasma levels (132-365 U/l)

iii. Bilirubin Estimation

Bilirubin estimation was done using Linear Chemicals S. L Spain kit method (Ref# 1111025). Serum was separated from the blood sample. Working reagent was prepared by mixing gently 1 part of RN and 4 parts of RT. Three tubes were taken and labelled reagent blank, sample blank and sample. Reagent blank contained 100 µL of water, sample blank contained 100 µL sample and 1.0mL RT solution, Sample contained 100 µL Sample and 1.0 mL of working reagent. The tubes were mixed thoroughly allowed to stand for 2 minutes at room temperature. Absorbance of sample

blank was taken at 540nm against water and absorbance of sample was taken at 540nm against reagent blank.

Calculations were done using the formula:

$$\frac{A_{(Sample)}-A_{(Sample\ blank)}\ XC_{(CSal)}}{A(Cal)} = \text{mg/dL total bilirubin}$$

Reference values: Serum/ Plasma of Adults (up to 1.0mg/dL)

E. VIRAL RNA PURIFICATION

Viral RNA purification was carried out using QuickSpin ® Viral Nucleic Acid Purification kit (Aj Roboscreen, Germany, CAT# NAP-01-03-050).

Reagent preparation:

Lysis buffer and wash buffer 2 were prepared before proceeding for viral RNA purification protocol. Lysis buffer was prepared by adding the carrier RNA contents to the whole lysis buffer bottle. Wash buffer 2 was prepared by adding 50mL ethanol to the whole bottle of concentrated wash buffer 2.

Protocol:

a) Virus Lysis

A 600 µL volume of lysis buffer (carrier RNA added) was added to 150 µL serum sample. Mixing was done by pipetting up and down and vortexing well. Incubation was done at 70ºC for 5 min.

b) Adjusting Binding Conditions

Then 600 µL volume of ethanol (absolute) was added to the clear lysis solution. Mixing was done by vortexing for 10-15 sec.

c) Binding Viral RNA

Binding columns were placed in 2ml collection tubes and 700 µL of lysed sample was loaded on it. Centrifugation was done at 8,000 x g for 1 min. The residual lysis solution was again reloaded on to the binding column. Centrifugation was done at 8,000 x g for 1 min. The flow-through was discarded. The binding column was placed into new 2 ml collection tube.

d) Washing and Drying Silica Membrane:

i. 1st Washing

First washing was done by adding 500 µL wash buffer 1 on to the column. Centrifugation was done at 8,000 x g for 1 min. The flow-through was discarded.

ii. 2nd Washing

Second washing was done by adding 600 µL wash buffer 2 on to the column. Centrifugation was done at 8,000 x g for 1 min. The flow-through was discarded.

iii. 3rd Washing

Third washing was done by putting the spin column in new 2ml collection tube and adding 200 µL wash buffer 2 on to the column. Centrifugation was done at 11,000 x g for 5 min to completely remove

wash buffer 2 supplemented with ethanol. The flow-through was discarded.

e) Viral Nucleic Acid Elution

The spin column was then placed into a new 1.5ml sterile microcentrifuge tube. Then 50 µl 70ºC preheated RNAase-free water was added to the column and incubated for 2 min. Centrifugation was done at 11,000 x g for 1 min.

F. GENOTYPE OF THE HCV

a) AMPLICOR® HCV Test, v2.0: Reagent Preparation

1. The number of reactions tubes required were taken including for specimen as well as control. The reaction tubes were placed in MicroAmp tray and locked in place with the help of retainer.
2. Working master mix was prepared by adding 100µl HCV Mn2+, v2.0 to single vial of HCV MMX,v2.0. Then 100 µL HCV Mn2+, v2.0 was added to HCV MMX, v2.0 entire vial. The tubes were recaped and mixed thoroughly by inverting 10-15 times. The remaining HCV Mn2+,v2.0 was discarded. Working master mix was stored at 2-8ºC and used subsequently with in 4 hours of preparation.
3. Then 50 µL of working master mix was added in each reaction tube.
4. The tray containing working master mix and capped reaction tubes were placed in a resealable plastic bag. The bag was sealed securely.

b) AMPLICOR® HCV Test, v2.0: Specimen and Control Preparation for

1. The 70% ethanol (15mL) was prepared that was enough for 12 tests.
2. The tubes were labeled for specimens, HCV(-)C and HCV(+)C.

3. Working lysis reagent was prepared by vortexing HCV IC, v2.0 for 10-15 sec. For each batch i.e., 12 specimens and controls, 100 µL HCV IC, v2.0 was added to the single bottle oh HCV LYS, v2.0 and mixed well.
4. Then 400 µL of working lysis reagent was added to each of the labeled tubes and the tubes were capped.
5. Controls were prepared as follows
    i. NHP, HCV(-)C,v2.0 and HCV(+)C, v2.0 were vortexed for 5-10 sec.
    ii. Then 200 µL NHP was added to both of the control tubes. Tubes were capped and vortexed for 3-5sec.
    iii. Then 20 µL of HCV(-)C,v2.0 was added to the tube having "HCV(-)C" label containing working lysis reagent and NHP. Tubes were capped and vortexed for 3-5sec.
    iv. Then 20 µL of HCV(+)C,v2.0 was added to the tube having "HCV(+)C" label containing working lysis reagent and NHP. Tubes were capped and vortexed for 3-5sec.
6. A 200 µL of each specimen was added to their respective labeled tubes containing working lysis buffer. Tubes were capped and vortexed for 3-5sec.
7. The sample and control tubes were incubated at about 60 ºC for 10 min. The tubes were then vortexed for at least 10 sec.

8. The caps were removed from the tubes, 600 µL isopropanol (100%, room temperature) was added to each tube. Tubes were recapped, vortexed for 3-5 sec and incubated for 2 min at room temperature.
9. Orientation of each tube was marked and placed in microcentrifuge with orientation mark facing outwards. The pellet formed will align orientation mark. Centrifugation was done for 15 min at 12,500-16,000xg at room temperature.
10. Supernatant was discarded.
11. Then 1.0mL 70% ethanol was added to each tube. The tubes were recapped and vortexed for 3-5sec.
12. The tubes were again centrifuged for 5 minutes with the same procedure as mentioned in step 9.
13. Supernatant was discarded.
14. The tubes were recapped and centrifuged for 3-5sec at maximum speed.the supernatant was removed carefully.
15. Then 200 µL HCV DIL, v2.0 was added to each tube. Pellet was dissolved using pipette and vigorous vortexing for 10 sec. the processed tubes were subjected to amplification with in 3 hours preparation.
16. A 50 µL of sample (specimen as well as control) were added to each reaction tube that contains working master mix. Reaction tubes were capped and sealed using MicroAmp Cap Installing Tool.
17. The position of each tube was recorded

c) AMPLICOR® HCV Test, v2.0: Reverse Transcription and Amplification

1. The tray was placed in thermal cycler block.
2. GeneAmp PCR System 2400 was programmed as follows:

Program	Temp	Time
HOLD Program	50 ºC	5min
HOLD Program	62 ºC	30 min
CYCLE Program	90 ºC	10 sec
	58 ºC	25 sec
HOLD Program	91 ºC	Not to exceed 3 hours

3. The tray was removed thermal cycler during the final HOLD program.
4. The caps were removed from the reaction tubes, 100µL [1] DN was added to the reaction tubes and mixed by pipetting up and down. Incubation was done at room temperature for 10 min to allow denaturation completely.
5. The reaction tubes were then saved at room temperature to process with in 2 hours for AMPLICOR® HCV Test, v2.0 detection.

d) AMPLICOR® HCV Test, v2.0: Detection

1. All reagents were warmed to room temperature
2. Water bath was warmed at 50ºC
3. Working hybridization buffer was prepared as follows: 62.5mL SSPE was added to 432.5mL distilled water and mixed well. Then 5mL SDS

was added and mixed well. Working hybridization buffer was stored at room temperature.

4. Working stringent wash buffer was prepared as follows: 25mL SSPE was added to 965mL distilled water and mixed well. Then 10mL SDS was added and mixed well. Working stringent wash buffer was stored at room temperature.
5. Working ambient wash buffer was prepared as follows: 37.5mL SSPE was added to1447.5mL distilled water and mixed well. Then 15mL SDS was added and mixed well. Working ambient wash buffer was stored at room temperature.
6. Working citrate buffer was prepared as follows: 50mL CIT was added to450mL distilled water and mixed well. Working citrate buffer was stored at room temperature.
7. Working hybridization buffer and working stringent wash buffer were warmed to 50ºC.
8. Shaking water bath was pre-warmed at 55ºC and set at 60 rpm shaking speed.
9. HCV strips were taken using clean forceps.
10. HCV strips were labeled using water proof ink pen
11. Strips were placed in the wells of 24 well tray with probe lines facing upward
12. Then 4mL working hybridization buffer was added to each well containing labeled strip.

13. Then 100µL denatured amplicon was added to each well containing labeled strip. The tray was rocked gently between each addition.
14. The tray was covered with lid placed in 55ºC and allowed to hybridize for 20 min at 60 rpm shaking.
15. Working conjugate was prepared as follows: 10µL SA-HRP was added to 5mL pre-warmed stringent wash buffer for each strip tested.
16. The tray was removed from shaking water bath. Working hybridization buffer was removed.
17. Then 4mL of working ambient wash buffer was added to each well of typing tray. Typing tray was gently rocked 3-4 times to rinse strips and working ambient wash buffer was removed.
18. Then 4mL working conjugate was added to each well of the typing tray. The typing tray was covered with lid and incubated back to water bath at 55ºC and 60 rpm shaking speed for 20 min.
19. The tray was removed from shaking water bath. Working conjugate was removed.
20. Then 4mL of working ambient wash buffer was added to each well of typing tray. Typing tray was gently rocked 3-4 times to rinse strips and working ambient wash buffer was removed.
21. Then 4mL of working stringent wash buffer was added to each well of typing tray. The typing tray was covered with lid and incubated back to water bath at 55ºC and 60 rpm shaking speed for 12 min.

22. The tray was removed from shaking water bath. Working stringent wash buffer was removed.
23. Then 4mL of working ambient wash buffer was added to each well of typing tray. Tray was incubated at room temperature orbital shaker, 60 rpm shaking speed for 5 min.
24. The tray was removed from orbital shaker. Working ambient wash buffer was removed.
25. Then 4mL of working citrate buffer was added to each well of typing tray. Tray was incubated at room temperature orbital shaker, 60 rpm shaking speed for 5 min.
26. Working substrate was prepared as follows: 4mL of SUB A was added to 1mL SUB B (per strip to be tested) and mixed well and stored at room temperature.
27. The tray was removed from orbital shaker. Working citrate buffer was removed.
28. Then 4mL of working substrate was added to each well of typing tray. Tray was incubated at room temperature orbital shaker, 60 rpm shaking speed for 10 min.
29. The tray was removed from orbital shaker. Working substrate was removed.
30. Then 4mL of distilled water was added to each well of typing tray. Tray was incubated at room temperature orbital shaker, 60 rpm shaking speed for 5 min.

31. The tray was removed from orbital shaker. Distilled water was removed.
32. Then 4mL of distilled water was added to each well of typing tray. Tray was incubated at room temperature orbital shaker, 60 rpm shaking speed for 5 min.
33. The tray was removed from orbital shaker. Distilled water was removed.
34. Then 4ml of distilled water was added to each well of typing tray. Strips were stored (for up to single day) in water at room temperature until interpretation.

G. VIRAL LOAD

HCV Viral load was estimated using RoboGene® HCV RNA Quantification Kit (Aj Roboscreen, Germany (Cat # 0207200162)). All the RT-PCR procedure was conducted at ice.

Preparation of 25x reagent mix (25x HCV_D4)

- A 40µL volume of PCR grade water was added to lyophilized HCV_D4 reagent mix vial and the lid was closed. The vial was incubated for 20 min at 37ºC. Vortexing was done for 3 sec and centrifugation was done at full speed for 5 sec.

REAGENT	VOLUME (µL)/ REACTION	FINAL CONCENTRATION
PCR grade water	3.5	
2x Reaction mixture	12.5	1x, 3mM Mg-sulphate
Mg-sulphate solution, 50mM	2.0	4.0mM
	results in final concentration 7.0mM of Mg-sulphate	
25x HCV_D4	1.0	1x
RT-PCR Enzyme Mix	1.0	1x

- Vortexing was done for 3 sec to mix. Centrifugation was done at full speed for 5 sec.
- The sample tubes (HCV_D2_LP) and quatification standard (HCV_D3_LP) were identified carefully and placed onto rack.
- Then 20 µL master mix (1x) was added to sample tubes as well as the quantification standard tubes.
- Then 5.0µL PCR grade water was added to sample tubes and quantification standard tubes making the volume not exceeding final reaction volume 25µL.
- 5.0 µL of isolated RNA was added to the samples tubes respectively making the volume not exceeding final reaction volume 25µL.
- The tubes were covered with caps. The sample and quantification standard strips were covered carefully.

- Centrifugation was done for 1 min at 200x g.
- The strips were carefully placed into the real time instrument block. The wells were selected that contained standards, non-template controls and samples. The text was entered identifying sample/ standard and number to represent quantity of standard.
- The setup was run as follows:

CLYCLING CONDITIONS FOR HCV RNA QUANTIFICATION

Step	**Temperature**	**Time**	**Repeat**
Reverse transcription	55 ºC	30 min	1
Taq Activation	95 ºC	2 min	1
Melting	95 ºC	30 sec	
Stem formation, fluorescence detection (FAM; JOE/VIC)	45 ºC	30 sec	45
Annealing/ synthesis	57 ºC	1 min	

Run time: ~2.5 hours

Data Analysis:

HCV RNA was estimated on the basis of C_T values for HCV RNA sample and standard curve resulting from quantification standards analysis and assay specific calibration co-efficient. HCV RNA concentration was expressed in IU/mL.

H. VIRAL HEPATITIS TESTS:

a. HAV ELISA Based Detection

Reagents Preparation: All components of IgM antibody to Hepatitis A virus ELISA kit were brought to room temperature (18-30◦C) before use. Reagents were shaken gently. Wash buffer was checked for any crystals, if found, were resolubilized by warming at 37 ◦C. The stock wash buffer was diluted with 1:20 ration with distilled/ deionized water.

Diluting the Sample: The specimen was diluted with 1:1000 ratio using normal saline.

Adding Sample: The 100 µl sample, positive and negative controls were added to each well.

Incubating the sample: The plate was covered with plate cover and incubated at 37 ◦C temperature for 20 minutes

Washing: After incubation, the plate cover was discarded and each well was washed 5 times with diluted wash buffer. The microwells were soaked for 30-60 seconds each time. After final washing step, the plate was turned down onto blotting paper and tapped to remove any residual liquid.

Adding Conjugate: A 100 µl OF HRP conjugate reagent was added to each well

HRP Conjugate Incubation: The plate was then covered with plate cover and incubated at temperature 37 ◦C for 40 minutes.

Washing: The plate cover was removed and discarded, liquid was apirated and each well was washed 5 times with wash buffer. After the final wash, the plates were turn down and tapped to remove any residual liquid.

Coloring: A 50 µl Chromogen A and 50 µl Chromogen B were added to each well including blank, mixed by tapping gently the plate. The plate was then incubated for 15 minutes at temperature 37 ◦C avoiding light. The chromogen solutions and HRP conjugate enzymatic reaction produced blue color in HAV-IgM positive samples and positive control.

Stopping Reaction: A 50 µl volume of stop solution was added to each well and mixed gently. Intensive yellow color developed in HAV-IgM positive samples and positive control.

Measuring the Absorbance: The absorbance was measured at 450nm against blank

b. HBV ELISA Based Detection

Reagents Preparation: All kit (HBsAg ELISA VERSION 1 Ref #: E0315) components were brought to room temperature (18-25◦C) before use. Reagents were shaken gently. The one volume of wash solution was diluted with 19 volume of distilled/ deionized water and mixed well using magnetic stirrer.

Measurement Procedure:

The 50 µl of negative and positive control and samples were added to respected wells. Then 50 µl of enzyme conjugate was added to each well. The plate was then put on

shaker to shake for 30 seconds to mix the liquid completely within the wells. Plate was covered and incubated for 30 minutes at 37 ◦C temperature. Then 350 µl of wash solution was added to each well, the plate was tapped and inverted to blot on blotting paper to remove any residual liquid. The process was repeated for 5 additional times. Then 50 µl of substrate A and then 50 µl of substrate B was added to each well. Gentle mixing was done for 15 seconds and incubation was done for 10 minutes at 37 ◦C in dark place without shaking. Then 50 µl stop solution was added to each well and mixed gently for color production. The color was then noted and absorbance at 450 nm was read in microplate reader.

c. HCV ELISA Based Detection

Reagents Preparation: All kit (Anti-HCV ELISA VERSION 1 Ref #: E0320) components were brought to room temperature (18-25◦C) before use. Reagents were shaken gently. "One part of wash solution was diluted with 19 parts of distilled/ deionized water and mixed well using magnetic stirrer".

Measurement Procedure:

A 100 µl of negative and positive control and sample diluents were added to respected wells. Then 10 µl of sample was added to the sample diluents containing wells. The plate was then put on shaker incubator for 30 seconds for complete mixing of the liquid within wells. Plate was covered and incubated for 30 minutes at 37 ◦C temperature. Then 350 µl of wash solution was added to each well, the plate was tapped and inverted to blot on blotting paper to remove any residual liquid. The

process was repeated for 5 additional times. Then 100 µl of enzyme conjugate was added to each well. The plate was covered and incubated for 30 minutes at 37 ◦C. Then 350 µl of wash solution was added to each well, the plate was tapped and inverted to blot on blotting paper to remove any residual liquid. The process was repeated for 5 additional times. Then 50 µl of substrate A and then 50 µl of substrate B was added to each well. Gentle mixing was done for 15 seconds and incubation was done for 10 minutes at 37 ◦C in dark place without shaking. Then 50 µl stop solution was added to each well and mixed gently for color production. The color was then noted and absorbance at 450 nm was read in microplate reader.

d. HDV ELISA Based Detection

Reagents Preparation: All kit (HDV Ab ELISA byDIA PRO Diagnostic Bioprobes, Srl., Italy) components were brought to room temperature (18-25◦C) before use. Reagents were shaken gently. The 20x wash buffer (60mL) was diluted up to volume of 1200mL with double distilled water and mixed gently before use.

Measurement Procedure:

A 100 µl of negative control, positive control, calibrator and samples were added to respected wells. The microplate was then incubated for 60 minutes at 37 ◦C. Then 350 µl of wash solution was added to each well, the plate was tapped and inverted to blot on blotting paper to remove any residual liquid. The process was repeated for 4 additional times. Then 100 µl of enzyme conjugate was added to each well. The plate was covered and incubated for 60 minutes at 37 ◦C. Then 350 µl of wash solution was

again added to each well, the plate was tapped and inverted to blot on blotting paper to remove any residual liquid. The process was repeated for 5 additional times. Then 100 µl of chromogen was added to each well. The microplate was then incubated for 20 minutes at room temperature. Then 100 µl of 0.3M Sulphuric acid was added to the wells. Then 50 µl stop solution was added to each well and mixed gently for color production. The color was then noted and absorbance at 450 nm was read in microplate reader.

e. HEV ELISA Based Detection

Reagents Preparation: All kit (HEV IgM ELISA byDIA PRO Diagnostic Bioprobes, Srl., Italy) components were brought to room temperature (18-25◦C) before use. Reagents were shaken gently. The 20x wash buffer (60mL) was diluted up to volume of 1200mL with double distilled water and mixed gently before use.

Measurement Procedure:

A 50 µl of neutralizing reagent was dispensed in all the wells of the samples. Then 100 µl of each Samples (i.e., 1000 µl sample diluents+10 µl of the sample) was added in the respected wells, 100 µl of negative and positive control in their respected wells. The microplate was then incubated for 60 minutes at 37 ◦C. Then 300 µl of wash solution was added to each well, the plate was tapped and inverted to blot on blotting paper to remove any residual liquid. The process was repeated for 4 additional times. Then 100 µl of enzyme conjugate was added to each well. The plate was covered and incubated for 60 minutes at 37 ◦C. Then 350 µl of wash solution was again added to

each well, the plate was tapped and inverted to blot on blotting paper to remove any residual liquid. The process was repeated for 5 additional times. Then 100 µl of chromogen/substrate mixture was added to each well. The microplate was then incubated for 20 minutes at room temperature. Then 100 µl of 0.3M Sulphuric acid was added to the wells. The color was then noted and absorbance at 450 nm was read in microplate reader.

2.6 EXPRESSION ANALYSIS

A. Primer Designing

Primers were designed specific to Interferon alpha receptor 1 and 2 sub-units and STAT 1 based on the previously known sequence as available on NCBI. Different bioinformatics tools including Oligo Calc: Oligonucleotide Properties Calculator (http://www.basic.northwestern.edu/biotools/oligocalc.html) and PrimerBank (http://pga.mgh.harvard.edu/primerbank/) were used for primer designing.

Gene	GenBank Accession	Primer	Sequence	Size (bps)
IFNAR1	NM_000629	Forward	5' AACAGGAGCGATGAGTCTGTC 3'	226
		Reverse	5' TGCGAAATGGTGTAAATGAGTCA 3'	
IFNAR2	NM_207585	Forward	5' ACAAGTGGCGGTGGCTATAC 3'	395
		Reverse	5' TCAGGATCCTCTGGGTCAAC 3'	
STAT1	NM_007315	Forward	5' GTCGGGGAATATTCAGAGCA 3'	198
		Reverse	5' TGATCACTCTTTGCCACACC 3'	

B. RNA Extraction and Reverse Transcription

GeneJET™ RNA Purification Kit (Cat # #K0731, Fermentas) was used to purify total RNA. Before starting RNA purification, buffers were prepared as indicated in the kit.

Prior to first use, 10 ml volume of ethanol (96-100%) was added to 40 ml of Wash Buffer 1 (concentrated) and 39 ml of ethanol (96-100%) was added to 23 ml Wash Buffer 2 (concentrated). After the addition of ethanol, the check box was marked on the bottle's cover in order to indicate the completed step. Before start of each RNA purification experiment, required amount of Lysis Buffer was supplemented with β-mercaptoethanol or DTT. To each 1 ml volume of Lysis Buffer, 20 µl of 14.3 M β-mercaptoethanol was added. Before each use, Lysis Buffer was checked for salt precipitation and if present, re-dissolved by warming solution at 37°C and then cooled down to 25°C before use. The whole procedure was carried out under aseptic conditions and wear gloves.

Total RNA Purification Protocol from Mammalian Tissue

Before starting:

The required amount of Lysis Buffer was supplement with β-mercaptoethanol i.e., 20 µl of 14.3 M β-mercaptoethanol was added to each 1 ml Lysis Buffer. The required amount of Proteinase K solution was prepared by diluting 10 µl Proteinase K to 590 µl TE buffer (APENDICE).

Procedure:

The sample was transferred to 1.5 ml Eppendorf and disrupted quickly by adding 300 µl of Lysis Buffer (supplemented with β-mercaptoethanol) and mixed thoroughly by vortexing for 10 seconds. A 600 µl volume of diluted Proteinase K i.e., 10 µl of Proteinase K in 590 µl of TE buffer was added to the lysate and mixed thoroughly by

vortexing. The tube was then incubated at 15-25°C for 10 min and centrifuge was done for 5 min (for lysate prepared from <10 mg of tissue sample) or 10 min (for lysate prepared from >10 mg of tissue) at a speed of ≥12000 x g. The supernatant was transferred into new sterile (RNase-free) microcentrifuge tube, and 450 µl ethanol (96-100%) was added and mixed by pipetting. Up to 700 µl of lysate was transferred to GeneJET RNA Purification Column that was inserted in a collection tube. Centrifugation was done for 1 min at a speed of ≥12000 x g. The flowthrough was discarded and the purification column was place back into collection tube. This step was repeated until all the lysate was transferred into the column and centrifuged. The collection tube was discard that contained the flow-through. GeneJET RNA Purification Column was placed into a new 2 ml collection tube and 700 µl Wash Buffer 1 (that was supplemented with ethanol) was added to the GeneJET RNA Purification Column and centrifugation was done for 1 min at a speed of ≥12000 x g. The flowthrough was discarded and purification column was placed back into the collection tube. Then 600 µl Wash Buffer 2 (also supplemented with ethanol) was added to the GeneJET RNA Purification Column and centrifugation was done for 1 min at a speed of ≥12000 x g. The flowthrough was discarded and purification column was placed back into the collection tube. The 250 µl Wash Buffer 2 was added to the GeneJET RNA Purification Column and centrifugation was done for 2 min at a speed of ≥12000 x g. The collection tube was emptied and residual solution was removed from the purification column by re-spinning the column at maximum speed for 1 minute. The collection tube was discarded which contained flow-through solution. The GeneJET RNA Purification Column was transferred to a new sterile RNase-free 1.5 ml

microcentrifuge tube and 50 µl of nuclease-free water was added to the center of the membrane of GeneJET RNA Purification Column and centrifugation was done for 1 min at a speed of ≥12000 x g to elute RNA. The purification column was discarded and purified RNA was stored at -80°C for cDNA synthesis.

C. First Strand cDNA Synthesis

The 1µg of total RNA isolated was then subjected to first strand cDNA synthesis using RevertAid Premium First Strand cDNA Synthesis Kit (#K1652, Fermentas). The kit components were thawed on ice and briefly centrifuged. The following reagents were added into a sterile (RNase free) tube placed on ice in the following order:

Components	**Amount**
Total RNA	1 µg
Oligo (dT)/ random hexamer primer/ gene-specific primer	1µl (100 pmol)
10 mM dNTP Mix	1 µl (0.5 mM final concentration)
Nuclease-free Water	15 µl
5X RT Buffer	4 µl
RevertAid Premium Enzyme Mix	1 µl
Total volume	20 µl

Tube was mixed gently and centrifuged. For oligo(dT)18 primer/ gene-specific primer, tube was incubated for 30 minutes at 50°C. For random hexamer primer used, the tube was incubated for 10 minutes at 25°C and then for 30 minutes at 50°C. The

reaction was terminated by heating the tube for 5 minutes at 85°C. The first strand cDNA synthesis reaction product was directly used for PCR.

D. PCR

The product from first strand cDNA synthesis reaction was directly used in PCR. Normally 5 µl of first strand cDNA synthesis reaction product was used as template for subsequent PCR reactions of 50 µl total volume. Phusion High-Fidelity PCR Master Mix (Cat # F-531S, Fermentas) was used to amplify IFNAR1.

Before opening, all the tubes were carefully mixed and centrifuged to ensure improve recovery and homogeneity. PCR reactions were always set up on ice. The components of the PCR were added in the order as follows:

Components	**Amount**
H_2O	Upto 50 µl
2x Phusion Master Mix	25 µl
Forward Primer	0.5 µM (Final Concentration)
Reverse Primer	0.5 µM (Final Concentration)
Template (cDNA)	5 µl
Total volume	50 µl

The following same cycling conditions were used to amplify IFNAR1, IFNAR2 and STAT1 sequence from cDNA previously synthesized except the annealing temperatures which were different for all the three sequences.

Cycle Step	Temperature	Time	Cycles
Initial denaturation	98°C	5 minutes	1
Denaturation	98°C	30 seconds	
Annealing	55°C for IFNAR1 50°C for IFNAR2 51°C for STAT1	30~45 seconds	30
Extension	72°C	30 seconds	
Final extension	72°C	10 minutes	1
	4°C	Hold	

E. Agarose Gel Electrophoresis

For visualizing the PCR products, 1.5 % agarose gel was used in tris-acetate-EDTA (TAE) buffer (Appendix II). The samples were electrophoresed for 50 - 60 minutes at 90 Volts. The gels were then stained in 0.1% ethidium bromide (EB) solution (Appendix II) and visualized using UV transilluminator. Bromophenol blue (BPB) 6x was always used as the loading/tracking dye. DNA bands were compared with 1Kb DNA ladder to locate the positions of bands.

2.7 STATISTICAL ANALYSIS

Data obtained was analyzed statistically using SPSS Version 20 for significance. Percentages were calculated for categorical data. Variables were

compared among groups using ANOVA, chi-square statistics or Student's *t*-test as appropriate. The values were considered significant at 95% confidence interval.

Chapter 3

RESULTS

PART-1: QUALITATIVE RESEARCH

HEPATITIS C VIRUS (HCV) AWARENESS

The awareness study included normal subjects (n=14), subjects with HCV infection history (n=65 i.e., 15 responders to standard interferon treatment and 50 non-responders to standard interferon treatment) and medical students (n=179). Normal subjects had age range between 21 and 59 years including 7 males and 8 females. Subjects with HCV infection history had age ranged between 25 and 74 years including 39 males and 26 females. Medical students had age range between 21 and 25 years including 57 males and 122 females of MBBS and BDS from 3rd year and 4th year of different medical colleges from Rawalpindi and Islamabad. The subjects who refused to take part in the study were not included in the final data analysis.

Regarding routes of HCV transmission, all subjects included in the study i.e., 94.97% medical students, 98.46% HCV infected subjects and 100% healthy subjects were well aware of the blood and its products as source of HCV infection (Table 3, Graph 1). Medical students (94.41%) and healthy subjects (92.86%) as compared to HCV infected subjects (55.38%) were well aware of the fact that injection drug users (IDU) are at greater risk of getting HCV as compared to patients with HCV infection history. There was confusion regarding sharing utensils as possible source of HCV

infection in all the groups i.e., only 51.40% medical students, 26.15% HCV infected subjects and 78.57% healthy subjects were aware.

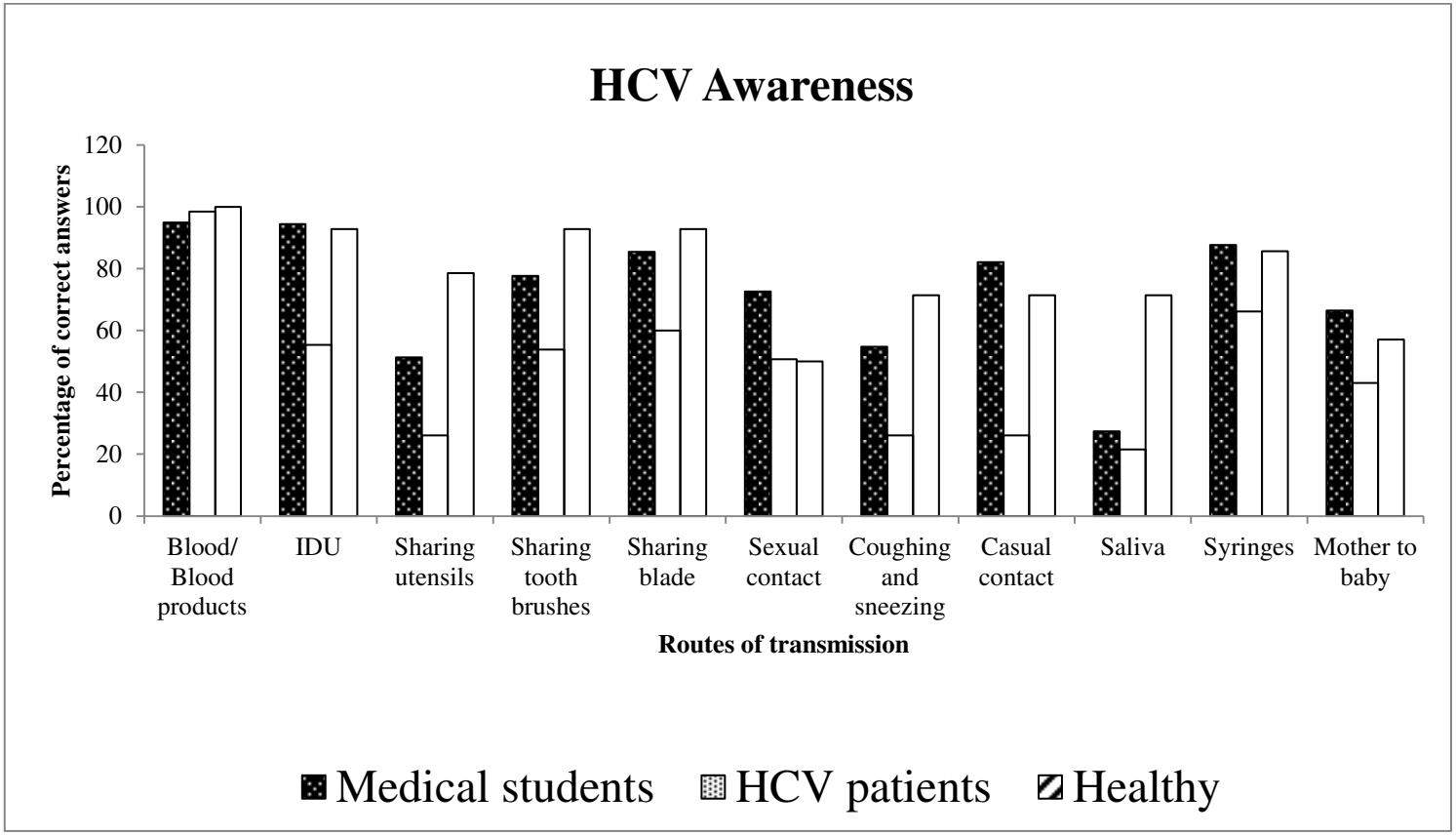
Medical students (77.65% and 85.47%) and healthy subjects (92.86%) were more knowledgeable regarding tooth brush and blade sharing as source of HCV infection compared to HCV infected subjects (53.85% and 60%) respectively. Regarding sexual contact as a source of HCV infection, 72.63% medical students, 50.77% HCV infected subjects and 50% healthy subjects were aware. Whereas for infected mother to baby as route of HCV transmission, 66.48% medical students, 43.08% HCV infected subjects and 57.14% healthy subjects were aware.

Subjects included in the study were also not well aware whether coughing & sneezing and saliva are sources of HCV infection or not i.e., only 54.75% medical students, 26.15% HCV infected subjects and 71.43% healthy subjects were aware that coughing sneezing is not source of HCV transmission whereas 27.37% medical students, 21.54% HCV infected subjects and 71.43% healthy subjects rule out saliva as route of HCV transmission. Regarding casual contact, patients had misperceptions (only 26.15% were aware) that once HCV is acquired, it will be transmitted to any other person who will be living with them. This was a major factor why most of the patients were depressed as relatives and friends were avoiding them. Most of the medical students (82.12%) and healthy subjects (71.43%) were aware that casual contact is not source of HCV transmission. Regarding re-use of syringe as a risk of HCV infection, 87.71% medical students, 66.15% HCV infected subjects and 85.72% healthy subjects were aware. Overall males were more knowledgeable as compared to females among all the groups included in the study.

Table 3: HCV Awareness Questionnaire and Medical Student's Response (Percentage of correct answers)

QUESTIONS	Medical students n (%)			Subjects with HCV infection history n (%)			Healthy subjects n (%)		
	Males (n=57)	Females (n=122)	Total (n=179)	Males (n=39)	Females (n=26)	Total (n=65)	Males (n=6)	Females (n=8)	Total (n=14)
Blood/blood products	57 (100)	113 (92.62)	170 (94.97)	39 (100)	25 (96.15)	64 (98.46)	6 (100)	8 (100)	14 (100)
Injection drug users	57 (100)	112 (91.80)	169 (94.41)	23 (58.97)	13 (50)	36 (55.38)	6 (100)	7 (87.5)	13 (92.86)
Sharing utensils	36 (63.16)	56 (45.90)	92 (51.40)	12 (30.77)	5 (19.23)	17 (26.15)	5 (83.3)	6 (75)	11 (78.57)
Sharing tooth brushes	57 (100)	82 (67.21)	139 (77.65)	22 (56.41)	13 (50)	35 (53.85)	6 (100)	7 (87.5)	13 (92.86)
Sharing of blade	56 (98.25)	97 (79.51)	153 (85.47)	26 (66.67)	13 (50)	39 (60)	6 (100)	7 (87.5)	13 (92.86)
Sexual contact	55 (96.49)	75 (61.48)	130 (72.63)	20 (51.28)	13 (50)	33 (50.77)	3 (50)	4 (50)	7 (50)
Coughing and sneezing	50 (87.72)	48 (39.34)	98 (54.75)	14 (35.90)	3 (11.54)	17 (26.15)	4 (66.67)	6 (75)	10 (71.43)
Casual contact	56 (98.25)	91 (74.59)	147 (82.12)	13 (33.33)	4 (15.38)	17 (26.15)	4 (66.67)	6 (75)	10 (71.43)
Saliva	25 (43.86)	24 (19.67)	49 (27.37)	12 (30.77)	2 (7.69)	14 (21.54)	5 (83.3)	5 (62.5)	10 (71.43)
Re-use of syringes	57 (100)	100 (81.97)	157 (87.71)	28 (71.79)	15 (57.69)	43 (66.15)	6 (100)	6 (75)	12 (85.72)
Mother to baby	57 (100)	62 (50.82)	119 (66.48)	16 (41.03)	12 (46.15)	28 (43.08)	3 (50)	5 (62.5)	8 (57.14)

Graph 1: Graphical presentation of the awareness level of the subjects included in the study



SOCIO-ECONOMIC STATUS (SES)

The cost for HCV diagnostics and treatment from different hospitals of Rawalpindi/ Islamabad was also approximately estimated in our study. The preliminary diagnostic tests for HCV (i.e., ELISA based detection, PCR based detection/ quantification, genotyping, liver function tests, etc) is above 200 USD i.e., equal to about average one month salary of a person in Pakistan. If the histopathology is included then it exceeds 300 USD. The standard IFN therapy costs more than 770 USD. But pegylated interferon costs above 2500 USD. The treatment response monitoring costs more than 200 USD. In this scenario, the salary does not permit the person to undergo routine basic diagnostic tests to timely diagnose any infection/ disease. Treatment costs along with other charges including doctor's fee, hospitalization and other expenses make the life of a person with average salary worst.

Socioeconomic status of the patients was determined by considering three markers i.e., education, employment/ occupation and income.

Educational level was categorized into 5 groups namely no-education, below matriculation, matriculation, graduation and post-graduation. About 92% of the males and ~80% of the females having HCV infection history either had no education or were metric/ below matric. About 67% males and ~75% females of healthy subjects were above matric in education. The education level was significantly different among groups ($p < 0.05$) as shown in Table 4 and Graph 2. The healthy subjects were significantly highly educated as compared to subjects with HCV infection history.

The subjects were also categorized based on the occupation. Among HCV infected patients including responders as well as non- responders, there were unemployed 11 individuals (10 males and 1 female) included in the study. Nine subjects were labors (all males), 6 healthcare staff persons (5 males and 1 female), 1 businessmen (male), 1 student (female), 1 teacher (female), 2 drivers (males), 11 government servants (all males), 1 shopkeeper (male) and 22 house wives. Among healthy subjects, 4 individuals were from health care staff (3 males and 1 female), 5 students (2 males and 3 females), 3 teachers (1 male and 2 female) and 2 house wives were also included (as shown in Table 5 and Graph 3.)

Income was classified PKR/ month in 4 categories: Level 1 (≤6000), 2 (6000-20,000), 3 (20-40,000) and 4 (>40,000). Most of the HCV infected subjects included in the study had income 20,000 PKR/ month or less whereas most of healthy subjects had income level 20,000 or above PKR/ month (Table). The income for unemployed persons was marked zero unless they indicated some other source e.g., property benefits (hiring/ renting etc). Similarly house wives income was marked based on their husband's income. The income difference among groups was also significant ($p < 0.05$) as shown in Table 6 and Graph 4.

RISK FACTORS FOR HCV INFECTION

In males with HCV infection history, visit to non-classified dentists and barber's shaving were the high risk factors involved in acquiring HCV infection, whereas in females, surgical operations and blood transfusions were the most common possible routes of acquiring HCV. None of the case had tattoos or organ transplant in

our study. The other risk factors for males and females observed in the study include contact with needle pricks, sharing syringes, tooth brushes, history of cuts, hospital admissions and history of injections. Razor sharing, kidney dialysis and IDU were found to be particularly associated with the males. Body piercing was only observed in females (Table 7).

Table 4: Educational level of the subjects included in the study

Education level	**Subjects with HCV infection History n(%)**				**Healthy Subjects n(%)**			
	Males (n=39)	Females (n=26)	Total (n=65)	P value	Males (n=6)	Females (n=8)	Total (n=14)	P value
No education	4 (10.26)	8 (30.77)	12 (18.46)	0.037	0 (0)	0 (0)	0 (0)	NS
Bellow Metric	9 (23.08)	4 (15.38)	13 (20)	0.448	0 (0)	1 (12.5)	1 (7.14)	0.369
Metric	23 (58.97)	9 (34.62)	32 (49.23)	0.054	2 (33.33)	1 (12.5)	3 (21.43)	0.347
Graduate	3 (7.69)	5 (19.23)	8 (12.31)	0.165	3 (50)	5 (62.5)	8 (57.14)	0.640
Post graduate	0 (0)	0 (0)	0 (0)	NS	1 (16.67)	1 (12.5)	2 (14.29)	0.825

Education level	No education	Bellow Metric	Metric	Graduate	Post graduate
Subjects with HCV infection History vs Healthy Subjects (P values)	0.081	0.253	0.0575	<0.001	0.002

Graph 2: Graphical presentation of the educational level of the subjects included in the study

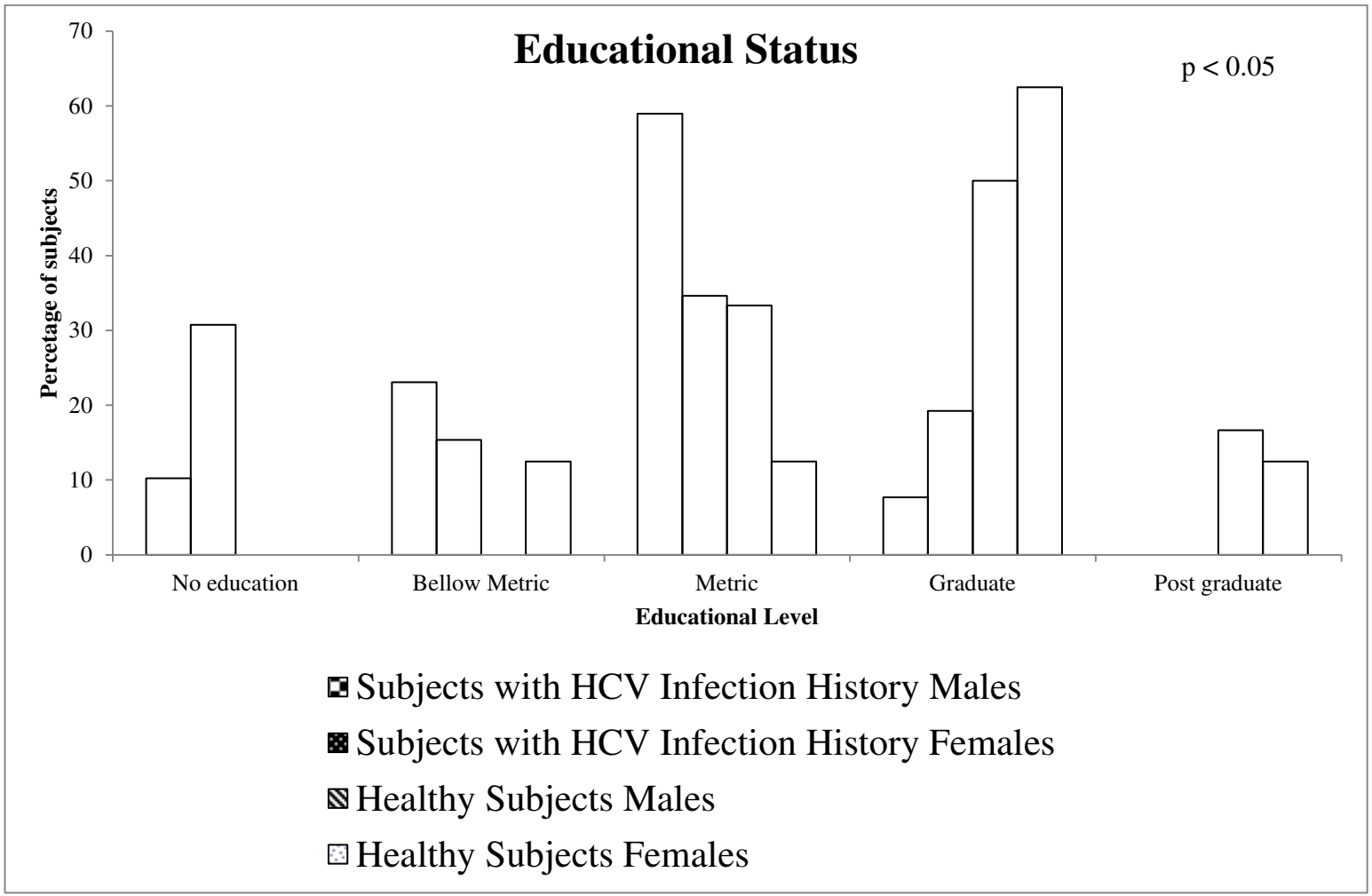


Table 5: Employment status of the subjects included in the study

	Subjects with HCV infection History n (%)			**Healthy Subjects n (%)**			**P values**
Employment status	Males (n=39)	Females (n=26)	Total (n=65)	Males (n=6)	Females (n=8)	Total (n=14)	
Unemployed	10 (25.64)	1 (3.85)	11 (16.92)	0 (0)	0 (0)	0 (0)	0.097
Labor	9 (23.08)	0 (0)	9 (13.85)	0 (0)	0 (0)	0 (0)	0.139
Healthcare staff	5 (12.82)	1 (3.85)	6 (9.23)	3 (50)	1 (12.5)	4 (28.57)	0.048
Businessman	1 (2.56)	0 (0)	1 (1.54)	0 (0)	0 (0)	0 (0)	0.640
Student	0 (0)	1 (3.85)	1 (1.54)	2 (33.33)	3 (37.5)	5 (35.71)	<0.001
Teacher	0 (0)	1 (3.85)	1 (1.54)	1 (16.67)	2 (25)	3 (21.43)	0.002
Driver	2 (5.13)	0 (0)	2 (3.08)	0 (0)	0 (0)	0 (0)	0.506
Government servants	11 (28.21)	0 (0)	11 (16.92)	0 (0)	0 (0)	0 (0)	0.097
Shopkeeper	1 (2.56)	0 (0)	1 (1.54)	0 (0)	0 (0)	0 (0)	0.640
House wife	N/A	22 (84.62)		N/A	2 (25)		0.001

Graph 3: Graphical presentation of the employment status of the subjects in the study

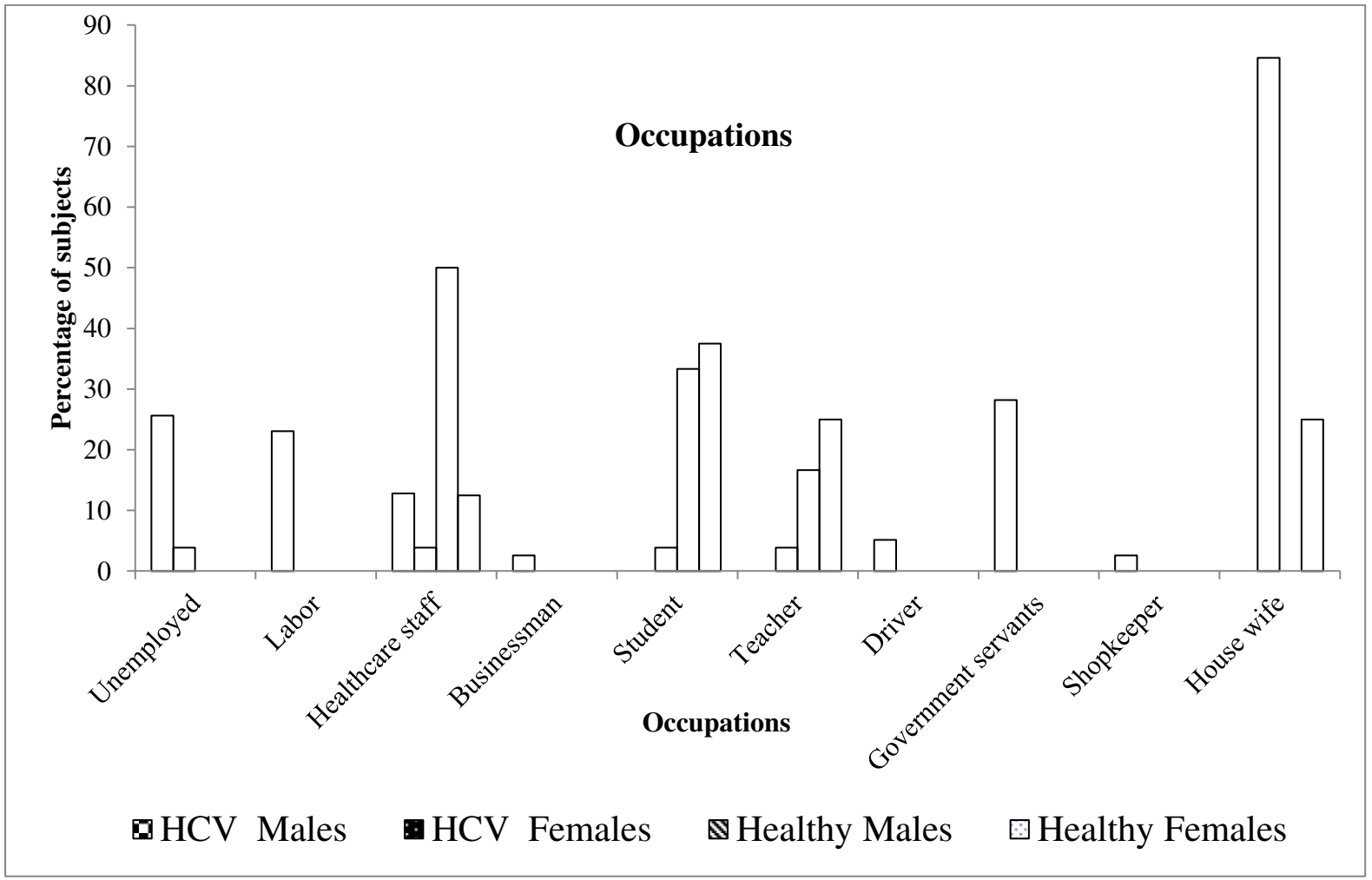


Table 6: Income status of the subjects included in the study

Monthly Income (in rupees)	**Subjects with HCV infection History n (%)**			**Healthy Subjects n (%)**			**p-value**
	Males (n=39)	Females (n=26)	Total (n=65)	Males (n=6)	Females (n=8)	Total (n=14)	
0 (No income)	6 (15.38)	0 (0)	6 (9.23)	0(0)	0(0)	0 (0)	0.237
1 (≤6000)	12 (30.77)	7 (26.92)	19 (29.23)	0 (0)	0 (0)	0 (0)	0.020
2 (6000-20,000)	9 (23.08)	13 (50)	22 (33.85)	2 (33.33)	2 (25)	4 (28.57)	0.703
3 (20-40,000)	10 (25.64)	6 (23.08)	16 (24.62)	3 (50)	5 (62.5)	8 (57.14)	0.016
4 (>40,000)	2 (5.13)	0 (0)	2 (3.077)	1 (16.67)	1 (12.5)	2 (14.29)	0.082

Graph 4: Graphical presentation of the income status of the subjects included in the study

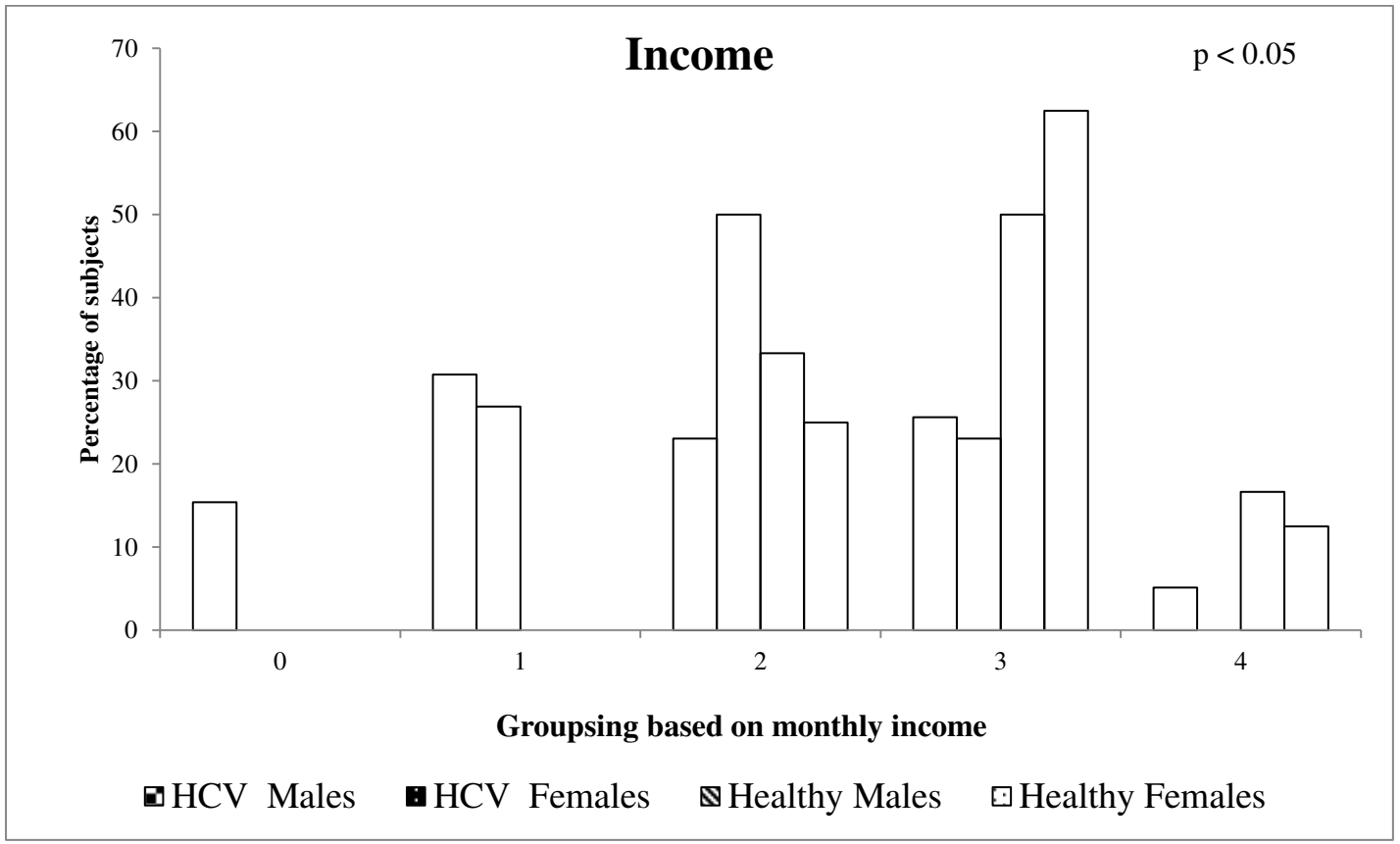


Table 7: Percentage of possible risk factors exposure in study subjects

Groups	Sex	Blood transfusion	Dental surgery classified	Dental surgery non-classified	Contact with blood/needle	Sharing syringes	sharing tooth brush	sharing razor	IDU
Subjects with HCV infection history	Males (n=39)	7 (17.95)	1 (2.56)	12 (30.77)	4 (10.26)	1 (2.56)	2 (5.13)	10 (25.64)	2 (5.13)
	Females (n=26)	7 (26.92)	1 (3.85)	2 (7.69)	1 (3.85)	1 (3.85)	1 (3.85)	0 (0)	0 (0)
Healthy normal subjects	Males (n=6)	1 (16.67)	2 (33.33)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Females (n=8)	0 (0)	1 (12.5)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Groups	Sex	Barber shave	Surgical operation	Blood loss due to accident	History of Cut/ Major injury	Ever admitted hospitals	body piercing	Injection history	Kidney dialysis
Subjects with HCV infection history	Males (n=39)	10 (25.64)	2 (5.13)	7 (17.95)	1 (2.56)	9 (23.08)	0 (0)	9 (23.08)	2 (5.13)
	Females (n=26)	0 (0)	17 (65.38)	0 (0)	2 (7.69)	18 (69.23)	26 (100)	2 (7.69)	0
Healthy normal subjects	Males (n=6)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	Females (n=8)	0 (0)	2 (25)	0 (0)	0 (0)	2 (25)	8 (100)	0 (0)	0 (0)

PART -11: QUANTITATIVE RESEARCH

The quantitative research was conducted including 80 subjects (15 normal, 15 responders to interferon treatment and 50 non-responders to interferon treatment). Their demographic as well as clinical parameters were analysed along with detection of liver expression of 3 genes IFNAR1, IFNAR2 and STAT. All the subjects included in the study were negative for ELISA based detection of HIV and viral hepatitis except group 3 which was only positive for HCV.

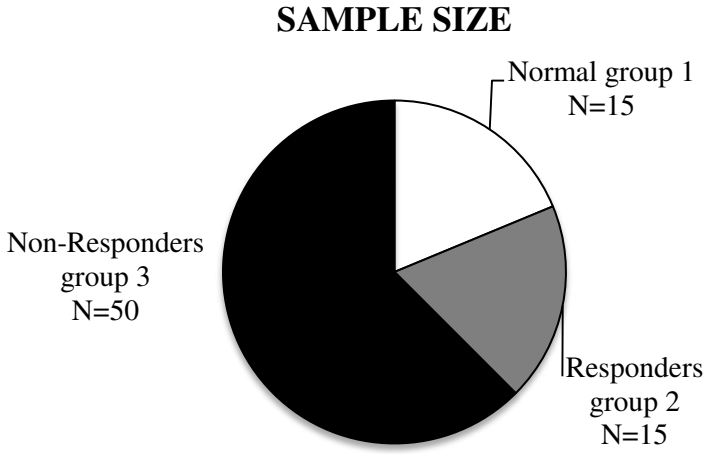


Fig 10: Subjects included in the study based on response to therapy

3.1 AGE

The study included 80 subjects with 15 normal, 15 responders to standard interferon treatment and 50 non-responders to standard interferon treatment. Normal subjects (group I) have age ranged between 21 and 59 years (mean of 36.6 ± 9.7

years), responders to standard IFN therapy (group II) have age ranged between 25 and 46 years (mean of 34.9 ± 6.8 years) and non- responders (group III) included in the study have age ranged between 29 and 74 years (mean of 42.0 ± 11.2 years). Age was significantly different among the three groups ($p = 0.032$). Mean age of non-responders was significantly higher than responders to IFN treatment ($p = 0.005$). There was no significant difference observed between the mean ages of normal vs responders ($p = 0.59$) and normal vs non-responders ($p = 0.07$) as shown in Table 8a and b, Graph 5.

3.2 SEX

Most of the patients non-responder to interferon treatment recruited randomly in the study were males (66%) i.e., 2:1 ratio of males as compared to females. Normal groups comprised of 46.7% males and 53.3% females and responders were 40% males and 60% females (Table 9 and Graph 6).

3.3 Marital Status

Most of the subjects as well as control subjects were married. Among the normal group, 66.7% subjects were married whereas 33.3% were unmarried. All subjects (100%) were married in control group 2 whereas 96% of the subjects included in the group 3 were married. The number of married subjects included in the study was significantly higher than single (Table 10, Graph 7).

3.4 Body Mass Index

BMI of normal subjects (group I) ranged between 20.78 and 26.49 (mean of 23.97 ± 1.76), responders to standard IFN therapy (group II) ranged between 21.53 and 25.77 (mean of 22.95 ± 1.18) and non- responders (group III) ranged between 20.18 and 34.67 (mean of 26.88 ± 3.20). Mean BMI of the non-responders falls in the overweight category. BMI was highly significantly different among the three groups ($p < 0.001$). Mean BMI of non-responders was significantly higher than responders to IFN treatment ($p < 0.001$) and normal healthy subjects ($p < 0.001$). No significant difference was observed between mean BMI values of normal and responders (Table 11a and b, Graph 8).

Table 8a: AGE: Group Comparison via ANOVA between Normal, Responders and Non-Responders

Groups	N	Mean	Std. Deviation	Minimum	Maximum	p-value
Normal	15	36.60	±9.66	21.00	59.00	0.032
Responders	15	34.93	±6.80	25.00	46.00	
Non-responders	50	42.04	±11.23	29.00	74.00	

Table 8b: AGE: Comparison between different groups along with their p-values

Groups	p-value
Normal v Responders	0.590
Normal v Non-responders	0.077
Responders v Non-responders	0.005

Graph 5: Age of the Subjects included in the study

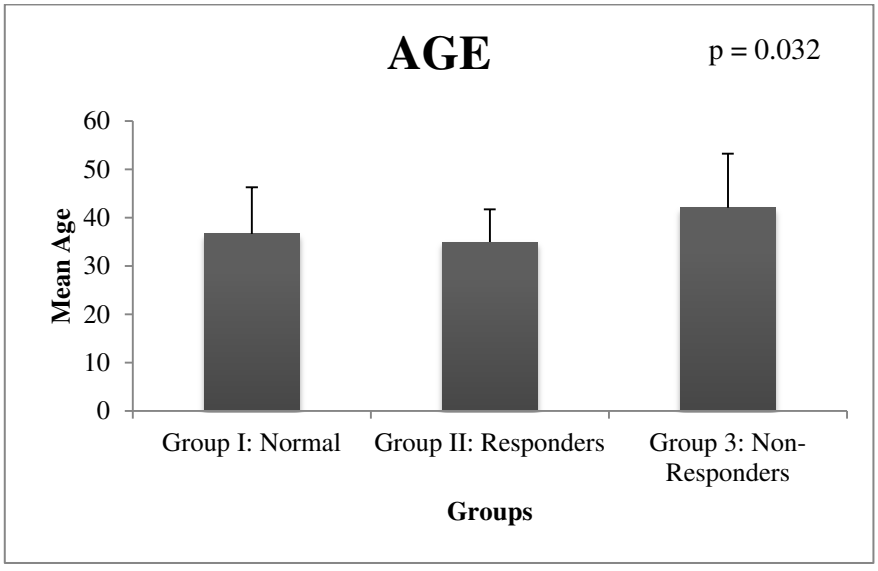


Table 9: Sex of the Subjects included in the study

Groups	N	Male	Female	p-value
1	15	7 (46.7%)	8 (53.3%)	0.130
2	15	6 (40%)	9 (60)	
3	50	33 (66%)	17 (34%)	

Graph 6: Sex ratio of the Subjects included in the study

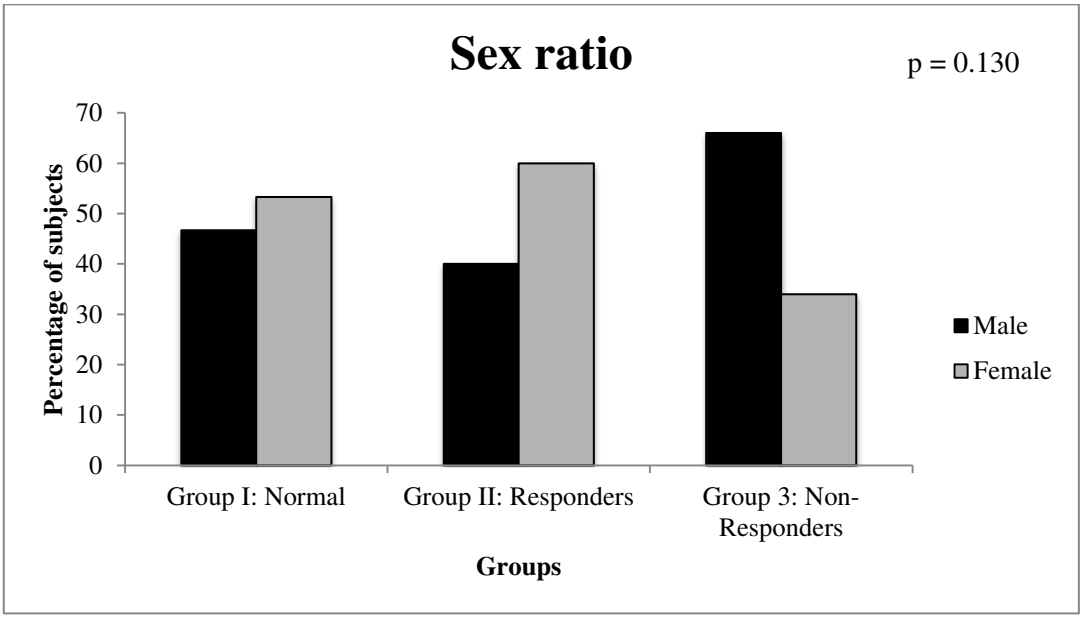


Table 10: Marital status of the Subjects included in the study

Groups	N	Married	Unmarried	p-value
1	15	10 (66.7%)	5 (33.3%)	< 0.001
2	15	15 (100%)	0 (0%)	
3	50	48 (96%)	2 (4%)	

Graph 7: Marital status of the Subjects included in the study

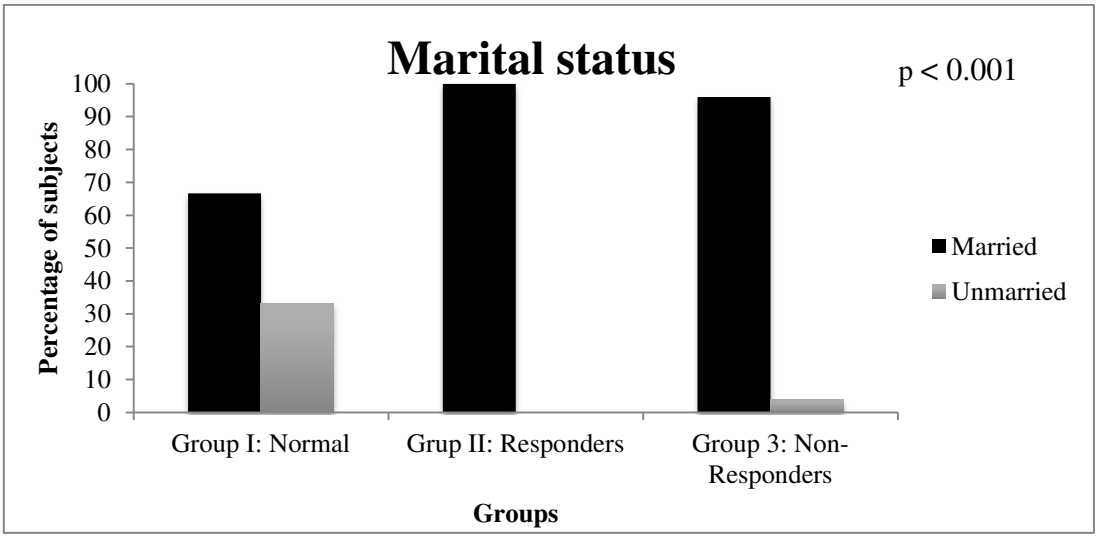


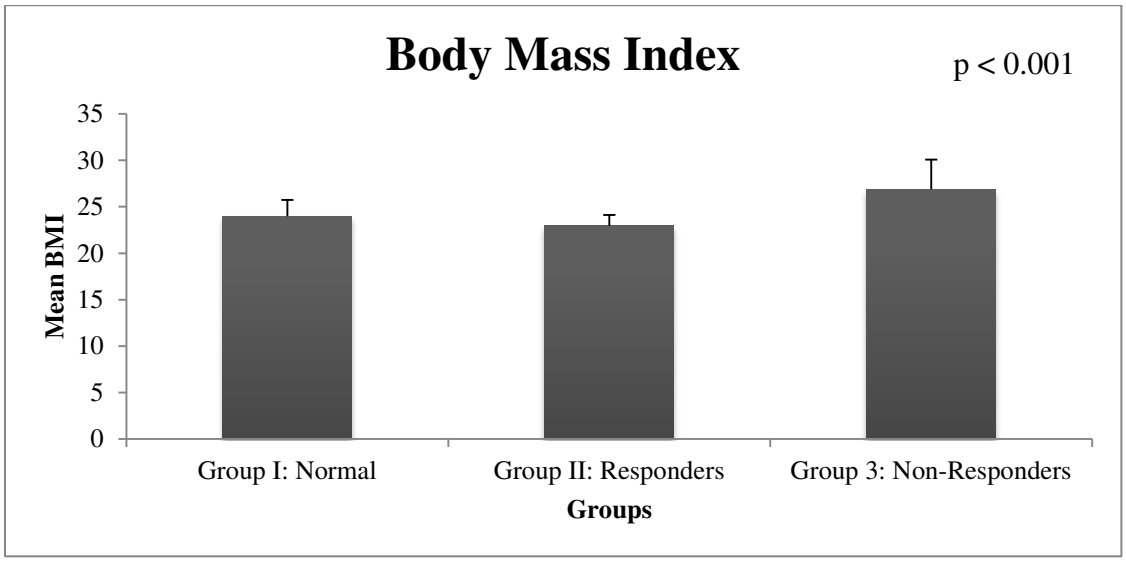
Table 11a: BMI: Group Comparison via ANOVA between Normal, Responders and Non-Responders)

Groups	N	Mean	Std. Deviation	Min.	Max.	p-value
Normal	15	23.97	±1.76	20.78	26.49	< 0.001
Responders	15	22.95	±1.18	21.53	25.77	
Non-responders	50	26.88	±3.20	20.18	34.67	

Table 11b: BMI: Comparison between different groups along with their p-values

Groups	p-value
Normal v Responders	0.072
Normal v Non-responders	< 0.001
Responders v Non-responders	< 0.001

Graph 8: Body Mass Index of the Subjects included in the study



3.5 LIVER FUNCTION TESTS

a. ALT

ALT (U/l) of normal subjects (group I) ranged between 18 and 41 (mean of 30.27 ± 7.87), responders to standard IFN therapy (group II) ranged between 22 and 56 (mean of 37.93 ± 9.10) and non- responders (group III) ranged between 20 and 340 (mean of 89.40 ± 66.60). ALT was highly significantly different among the three groups ($p < 0.001$). Mean ALT of non-responders was significantly higher than responders to IFN treatment ($p < 0.001$) and normal healthy subjects ($p < 0.001$). Significant difference was also observed between mean ALT values of normal and responders ($p = 0.020$) (Table 12a and b, Graph 9).

b. ALP

ALP (U/l) of normal subjects (group I) ranged between 104 and 356 (mean of 217.3 ± 69.4), responders to standard IFN therapy (group II) ranged between 165 and 348 (mean of 275.7 ± 52.6) and non- responders (group III) ranged between 56 and 569 (mean of 182.3 ± 91.7). ALP was significantly different among the three groups ($p = 0.001$). Though, the mean values of all the groups were in normal range. Mean ALP of responders was significantly higher than non-responders to IFN treatment ($p < 0.001$). Significant difference was also observed between mean ALP values of normal and responders ($p = 0.015$) but no significant difference was observed between normal and non-responders ($p = 0.124$). Significant difference was observed between responders and non-responders ($p < 0.001$) (Table 13a and b, Graph 10).

Table 12a: ALT (U/l): Group Comparison (ANOVA) between Normal, Responders and Non-Responders)

Groups	N	Mean	Std. Deviation	Min.	Max.	p-value
Normal	15	30.27	±7.87	18.00	41.00	< 0.001
Responders	15	37.93	±9.10	22.00	56.00	
Non-responders	50	89.40	±66.60	20.00	340.00	

Table 12b: ALT: Comparison between different groups along with their p-values

Groups	p-value
Normal v Responders	0.020
Normal v Non-responders	< 0.001
Responders v Non-responders	< 0.001

Graph 9: ALT of the Subjects included in the study

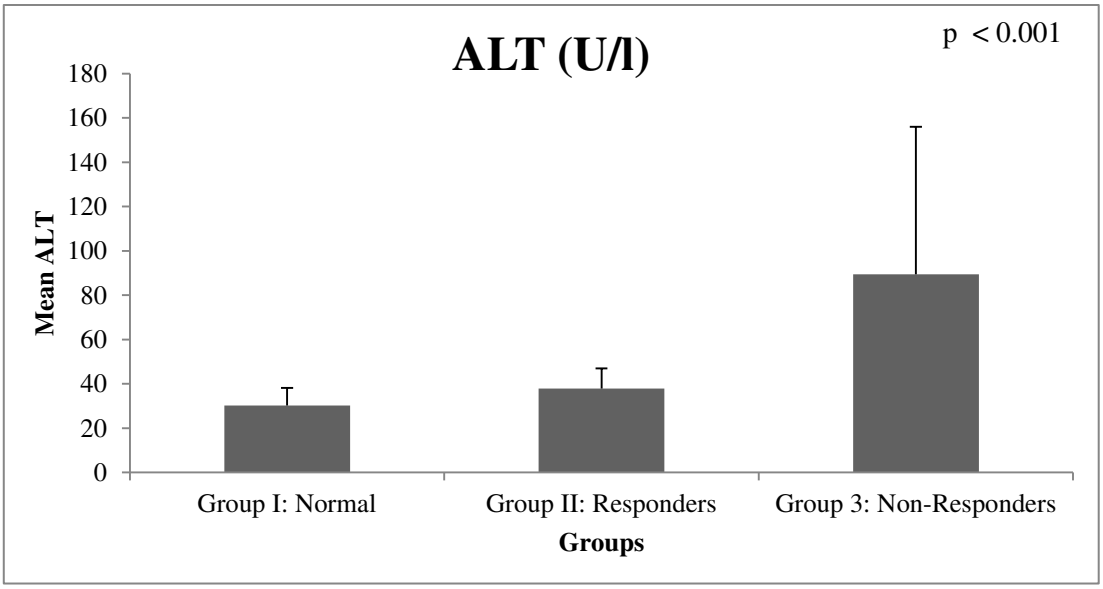


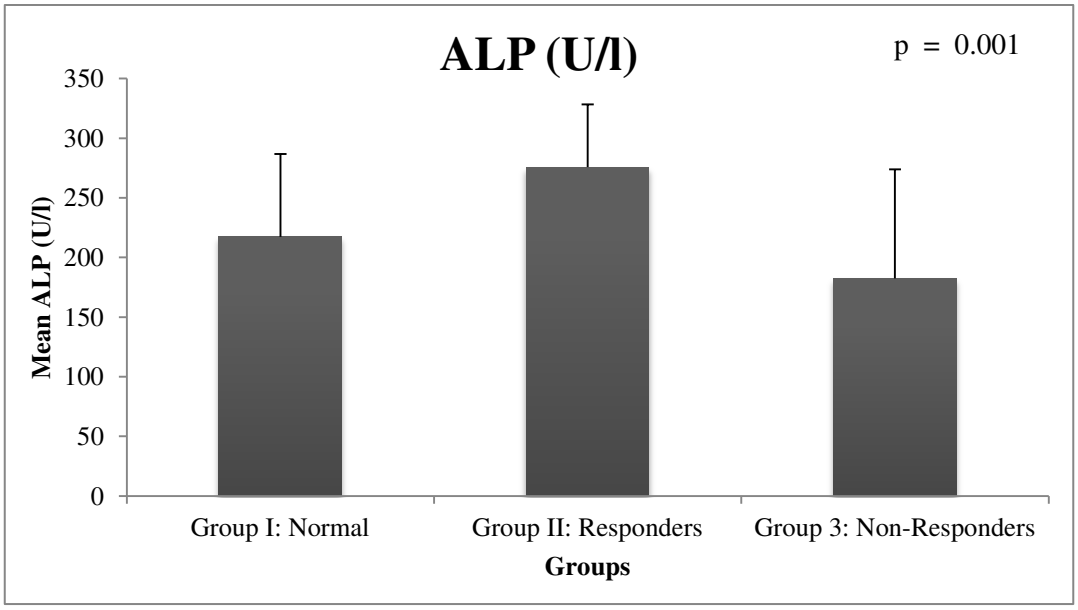
Table 13a: ALP: Group Comparison (ANOVA) between Normal, Responders and Non-Responders)

Groups	N	Mean	Std. Deviation	Min.	Max.	p-value
Normal	15	217.3	± 69.4	104.00	356.00	0.001
Responders	15	275.7	± 52.6	165.00	348.00	
Non-responders	50	182.3	± 91.7	56.00	569.00	

Table 13b: ALP: Comparison between different groups along with their p-values

Groups	p-value
Normal v Responders	0.015
Normal v Non-responders	0.124
Responders v Non-responders	< 0.001

Graph 10: ALP of the Subjects included in the study



a. BILIRUBIN

Bilirubin (mg/dL) of normal subjects (group I) ranged between 0.40 and 1.00 (mean of 0.73 ± 0.18), responders to standard IFN therapy (group II) ranged between 0.70 and 1.60 (mean of 1.03 ± 0.28) and non- responders (group III) ranged between 0.50 and 2.10 (mean of 1.36 ± 0.41). Bilirubin was highly significantly different among the three groups ($p < 0.001$). Mean Bilirubin of non-responders was significantly higher than normal ($p < 0.001$) and responders to IFN treatment ($p = 0.001$). Significant difference was also observed between mean Bilirubin values of normal and responders ($p = 0.003$) (Table 14a and 14b, Graph 11).

3.6 Liver Ultrasound

a. Liver Echo texture

The liver echo texture was normal in all subjects included in group I and II. In group III, 18 (36%) patients have liver cirrhosis (Table 15, Graph 12).

b. Spleen size

The spleen size was normal in subjects included in group I and II. The increase in spleen size was observed in 16% of the non-responders and 44.4% of the non-responders with cirrhotic liver (Table 16, Graph 13).

c. Portal Vein Diameter (PVD)

The PVD was normal in normal and responders groups but elevated in group III as compared to group I and II. PVD of normal subjects (group I) ranged between

0.90 and 1.10 (mean of 0.97 ± 0.07), responders to standard IFN therapy (group II) ranged between 0.90 and 1.10 (mean of 0.99 ± 0.07) and non- responders (group III) ranged between 0.90 and 1.40 (mean of 1.04 ± 0.14). PVD difference was insignificant among the three groups ($p = 0.095$). Mean PVD of non-responders was significantly higher than normal ($p = 0.012$) whereas in-significant as compared to responders to IFN treatment ($p = 0.068$). Mean PVD difference was also insignificant between normal and responders ($p = 0.443$) (Table 17a and 17b, Graph 14).

Table 14a: Bilirubin (mg/dL): Group Comparison (ANOVA) between Normal, Responders and Non-Responders)

Groups	N	Mean	Std. Deviation	Min.	Max.	p-value
Normal	15	0.73	± 0.18	0.40	1.00	
Responders	15	1.03	± 0.28	0.70	1.60	
Non-responders	50	1.36	± 0.41	0.50	2.10	< 0.001

Table 14b: Bilirubin: Comparison between different groups along with their p-values

Groups	p-value
Normal v Responders	0.003
Normal v Non-responders	< 0.001
Responders v Non-responders	0.001

Graph 11: Bilirubin of the Subjects included in the study

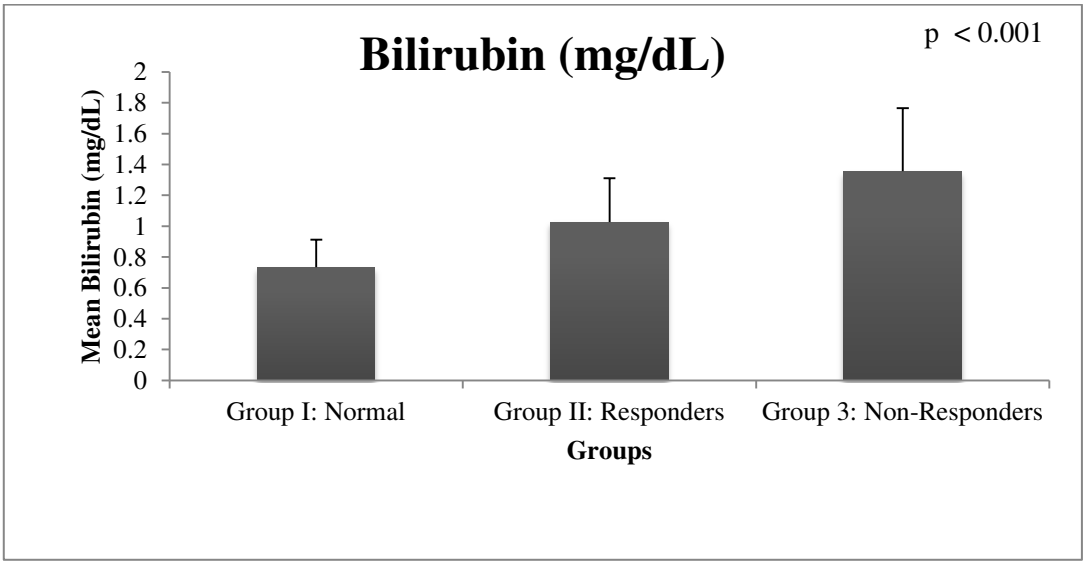


Table 15: Liver Echo texture of the Subjects included in the study

Groups	N	Liver Echotexture	n (%)	p-value
I	15	Normal	(15) 100	< 0.001
II	15	Normal	(15) 100	
III	50	Normal	32 (64)	
		Cirrhosis	18 (36)	

Graph 12: Liver Echo texture of the Subjects included in the study

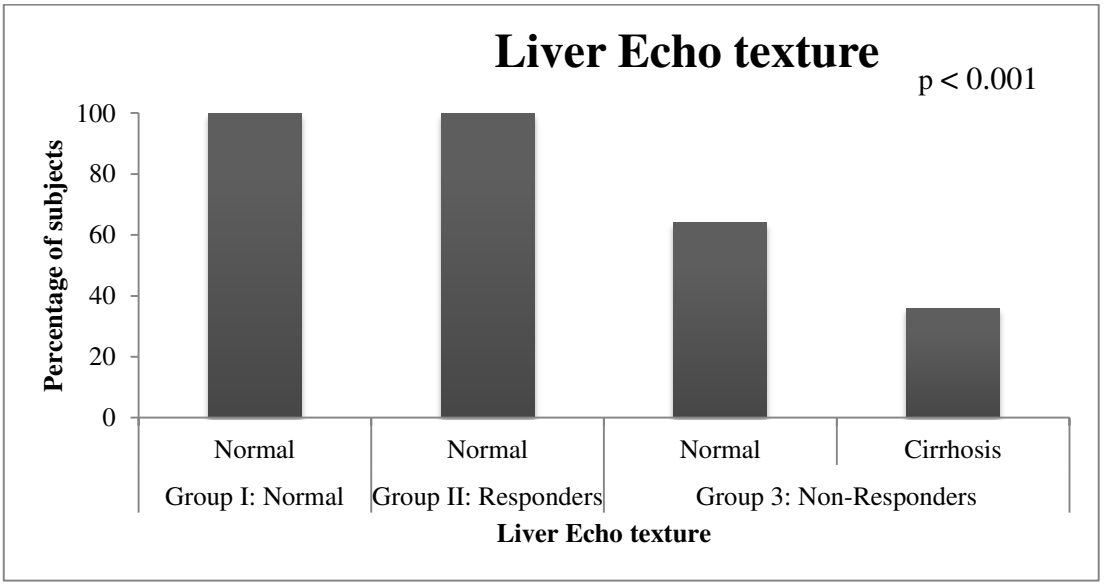


Table 16: Spleen size of the Subjects included in the study

Groups	N	Spleen size	n (%)	p-value
I	15	Normal	(15) 100	0.0694
II	15	Normal	(15) 100	
III	50	Normal	42 (84)	
		Increased	8 (16)	

Graph 13: Spleen size of the Subjects included in the study

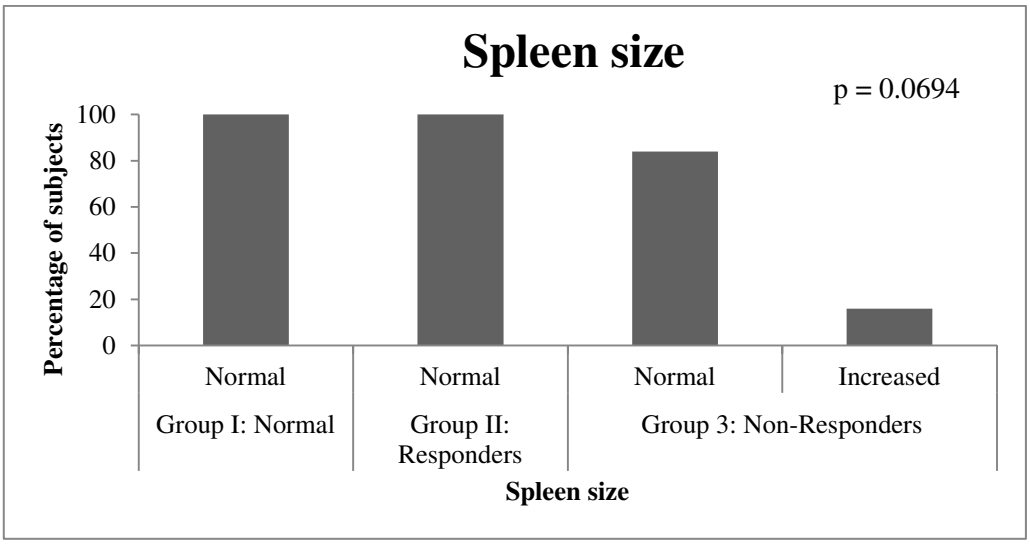


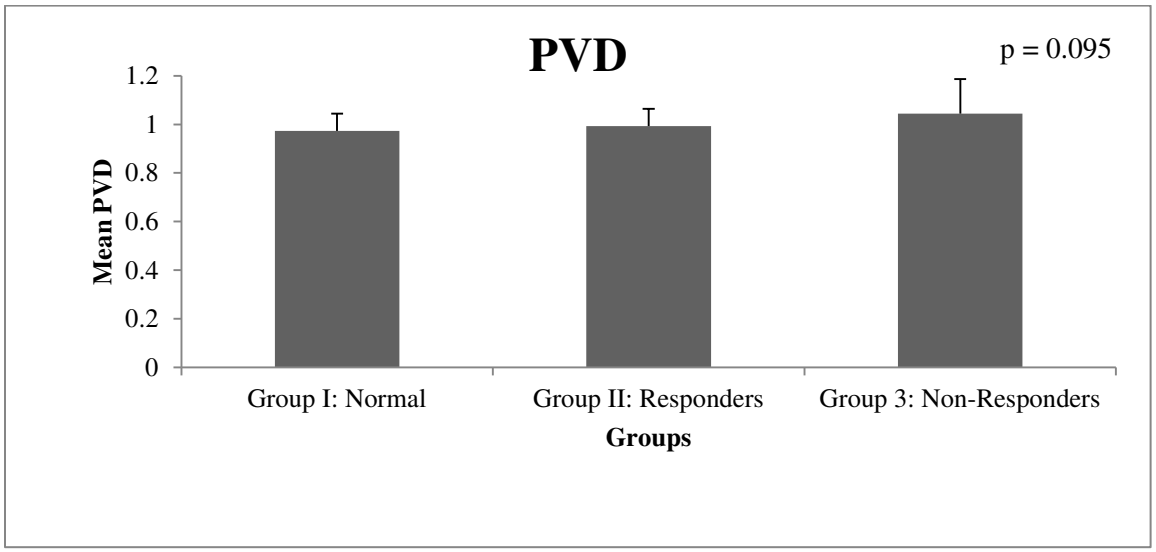
Table 17 a: Portal Vein diameter: Group Comparison (ANOVA) between Normal, Responders and Non-Responders)

Groups	N	Mean	Std. Deviation	Min.	Max.	P-value
Normal	15	0.97	± 0.07	0.90	1.10	0.095
Responders	15	0.99	± 0.07	0.90	1.10	
Non-responders	50	1.04	± 0.14	0.90	1.40	

Table 17 b: Portal Vein diameter: Comparison between different groups along with their p-values

Groups	P-value
Normal v Responders	0.443
Normal v Non-responders	0.012
Responders v Non-responders	0.068

Graph 14: PVD of the Subjects included in the study



3.7 EXPRESSION OF IFN RECEPTOR

Liver expression of IFNAR1, IFNAR2 and STAT1 was analyzed through qualitative PCR based detection method in HCV infected subjects non-responder to standard interferon treatment. It was found that IFNAR1, IFNAR2 and STAT1 were expressed in 46% (23/50), 94% (47/50) and 98% (49/50) of the liver tissues of the non-responders. Biopsy samples were not available for normal healthy subjects as well as those subjects who responded to standard interferon treatment. Hence whole blood was used for PCR standardization and detection. It was found that all of the normal healthy subjects and responders to IFN standard treatment were expressing IFNAR1, IFNAR2 and STAT1.

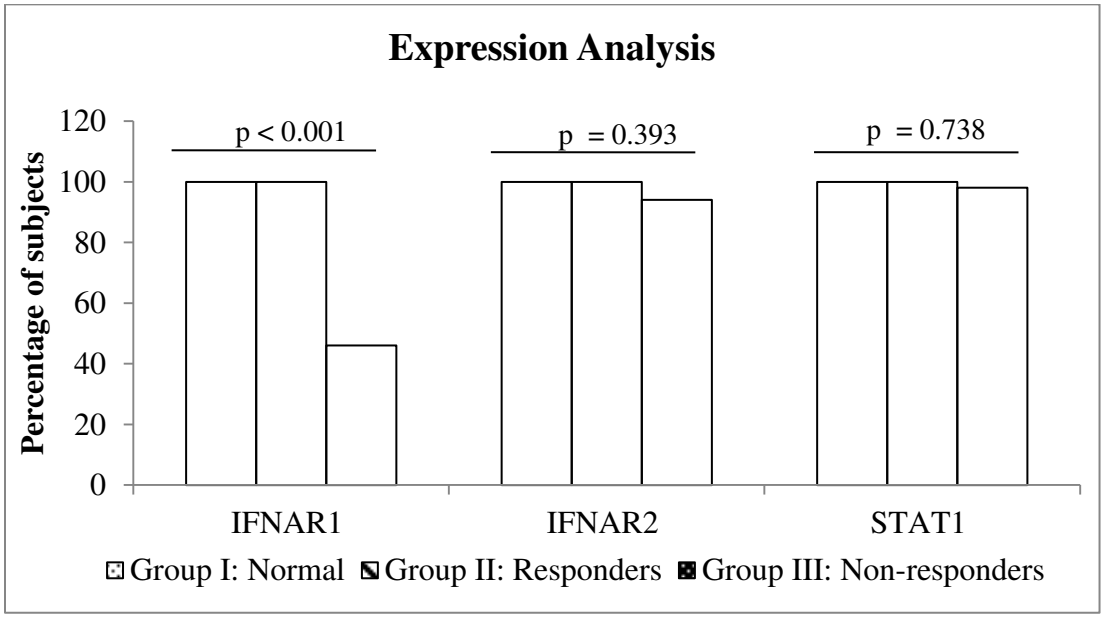
Eighteen patients (36%) among non-responders were cirrhotic and none of them was expressing IFNAR1. IFNAR2 was detected in 88.9% (16/18) and 96.9% (31/32) of cirrhotic and non-cirrhotic patients respectively. STAT1 was expressed in all patients except one cirrhotic patient (Table 18, Graph 15).

Among non-responders, the patients expressing IFNAR1 (23/50) i.e., 46% were designated group 3a and the 27/50 patients (54%) who did not express IFNAR1 were designated as group 3b. Significant difference was observed in age, BMI, ALT, ALP, bilirubin, portal vein diameter, viral load, liver histopathology (grade, stage and score) in patients who were negative for IFNAR1 expression as compared to positive one (summarized in table 21).

Table 18: IFNAR and STAT1 expression in the subjects included in study

Groups	N	IFNAR1 n (%)	IFNAR2 n (%)	STAT1 n (%)
Normal	15	15 (100)	15 (100)	15 (100)
Responders	15	15 (100)	15 (100)	15 (100)
Non-responders	50	23 (46)	47 (94)	49 (98)

Graph 15: IFNAR and STAT1 expression in the subjects included in study



3.8 VIRAL LOAD

The viral load was not detected in normal and responders groups but elevated in group III. Magnitude of viral load was divided into 3 catagories according to Jensen (Jensen et al., 2006):

1. Low (< 200,000 IU/mL),
2. Intermediate (200,001-600,000 IU/mL)
3. High (> 600,000 IU/mL)

Regarding viral load, a trend was observed with respect to IFNAR1 expression. Most of the patients with positive liver expression have lower viral load as compared to the subjects negative for IFNAR1 expression. Whereas patients lacking the expression of IFNAR1 negative expression group had higher viral load. Similarly the liver cirrhosis patients also had higher viral loads as compared to non-cirrhotic patients (Table 19, Graph 16).

3.9 Liver Histopathology

Liver histopathology data showed that in all patients of interferon therapy non-responders, inflammation ($p < 0.001$), fibrosis ($p < 0.001$) and overall score ($p < 0.001$) of liver histopathology status was significantly worse in cirrhosis as compared to non-cirrhosis patients and IFNAR1 expression negative subjects as compared to positive subjects (Table 20a and b).

Table 19: Viral load of the Subjects included in the study

Groups	Non-responders n=50 n (%)	IFNAR1 Positive n=23 n (%)	IFNAR1 Negative n=27 n (%)	Cirrhosis Patients n=18 n (%)	Non cirrhosis Patients n=32 n (%)
Low (< 200,000 IU/mL),	24 (48)	22 (95.65)	2 (7.41)	0 (0)	24 (75)
Intermediate (200,001-600,000 IU/mL)	11 (22)	1 (4.35)	10 (37.04)	6 (33.33)	5 (15.63)
High (> 600,000 IU/mL)	15 (30)	0 (0)	15 (55.56)	12 (66.67)	3 (9.38)

Graph 16: Viral load of the Subjects included in the study

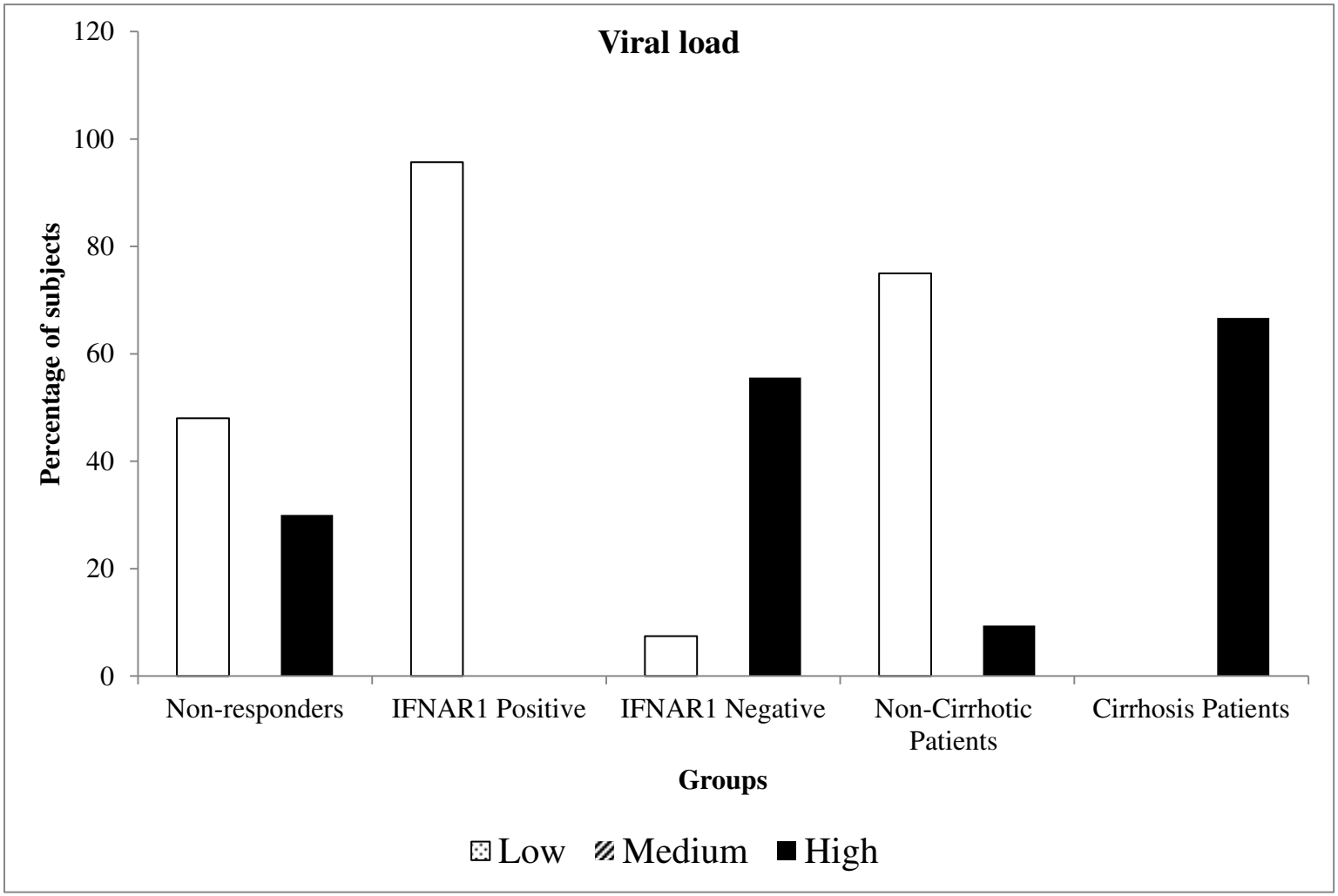


Table 20a: Liver Histopathology: Group Comparison via ANOVA

Groups	HAI	Mean	Std. Deviation	Min.	Max.
Non-responders n (50)	Grade	6.40	±2.03	4.00	10.00
	Stage	2.92	±1.40	1.00	6.00
	Score	9.32	±3.34	5.00	16.00
IFNAR1 Positive n (23)	Grade	4.78	±1.04	4.00	7.00
	Stage	1.91	±0.29	1.00	2.00
	Score	6.70	±1.11	5.00	9.00
IFNAR1 Negative n (27)	Grade	7.78	±1.60	4.00	10.00
	Stage	3.78	±1.40	2.00	6.00
	Score	11.56	±2.95	6.00	16.00
Non-cirrhosis Patients n (32)	Grade	5.06	±1.05	4.00	7.00
	Stage	1.94	±0.25	1.00	2.00
	Score	7.00	±1.11	5.00	9.00
Cirrhosis Patients n (18)	Grade	8.78	±0.73	8.00	10.00
	Stage	4.67	±0.69	4.00	6.00
	Score	13.44	±1.34	12.00	16.00

Table 20b: Liver Histopathology: Comparison between different groups included in the study

Groups		P-value
IFNAR1 Pos v IFNAR1 Neg	Grade	< 0.001
	Stage	< 0.001
	Score	< 0.001
Cirrhosis v Non cirrhosis	Grade	< 0.001
	Stage	< 0.001
	Score	< 0.001

Table 21: Comparison between Non responders IFNAR1 Positive group (3a) Vs Non Responders IFNAR1 Negative group (3b)

	Group	N	Mean	Std. Deviation	p-value
AGE	3a	23	37.09	±6.76	0.002
	3b	27	46.26	±12.60	
BMI	3a	23	25.88	±2.90	0.038
	3b	27	27.74	±3.25	
ALT	3a	23	52.87	±25.80	< 0.001
	3b	27	120.52	±74.91	
ALP	3a	23	150	±47.77	0.015
	3b	27	209.82	±110.53	
Bilirubin	3a	23	1.24	±0.39	0.054
	3b	27	1.46	±0.40	
USpvdia	3a	23	0.99	±0.09	0.011
	3b	27	1.09	±0.16	
Grade	3a	23	4.78	±1.04	< 0.001
	3b	27	7.78	±1.60	
Stage	3a	23	1.91	±0.29	< 0.001
	3b	27	3.78	±1.40	
Score	3a	23	6.70	±1.11	< 0.001
	3b	27	11.56	±2.95	

Chapter 4

DISCUSSION

Part I: Qualitative research

HCV Awareness

Hepatitis C virus (HCV) has infected ~170-180 million population worldwide (Houghton, 2009, Sulkowski et al., 2011, 2009) and Pakistan is amongst the high HCV prevalent countries (Martins et al., 2011).

The predictable increase in mortality rate 2-3 fold in next two decades as result of potential HCV-mediated cirrhosis development will make it signature for liver transplantation. These data suggest how HCV is significantly important global public health problem (Szabo et al., 2003). In order to control HCV infection, health education and counseling is important along with testing of high risk population (Zanetti et al., 2003). In this regard, evaluation of level of awareness regarding routes of HCV transmission among population is essential. Hence keeping in view the high burden of HCV in Pakistan, this study was conducted to investigate the awareness level among subjects with HCV infection history, normal healthy individuals and medical students who are at the start of their clinical rotation. There are number of well known risk factors i.e., blood and related products and hence subjects included in the study were more aware of the blood and its products as potent routes of HCV transmission. Other risk factors were also important but they were ignored. Our

finding was similar to the survey conducted by Tiftikci et al., to evaluate the awareness regarding routes of HCV transmission among healthcare staff (including first-year and last-year medical students, dentists, pharmacists and nurses), hepatitis C infected patients and their household contacts in Turkey. It was found that HCV transmission by blood/blood products was well aware among the subjects included in the study whereas the other means of HCV transmission were either over-estimated or under-recognized (Tiftikci et al., 2009). Similarly earlier studies conducted in different regions of Pakistan also show that HCV awareness level is not adequate in Pakistani population (Jamil et al., 2010, Janjua and Nizamy, 2004). We also observed same situation in subjects included in our study. Except the blood products as source of HCV transmission, other factors were either over-estimated or under-recognized. Medical students and healthy subjects were found to be more aware of the well known risks of acquiring HCV as compared to patients with HCV infection history. The awareness level among HCV infected subjects was alarmingly low and this is the reason why they were not timely aware of their infection and the lack of knowledge can seriously pose threats to infection transmission to their close contacts.

There was significant level of misconception observed in our study e.g., there is no evidence about association of casual contacts or sharing utensils with transmission of HCV (Lo Re and Kostman, 2005). Sneezing and coughing are also not the sources of HCV transmission (Arend, 2000). In a survey, 28% of the participants thought coughing or sneezing can transmit HCV from infected person to healthy one (Norton et al., 2014). In present study, we found subjects have misconceptions that

sharing utensil, casual contact and coughing and sneezing are the risks for acquiring HCV. This is alarming situation as if the medical students are not aware of the fact that casual contact/ sharing utensils/ sneezing coughing have not been associated with the HCV transmission then how will they treat the HCV infected patients efficiently. Similarly the knowledge is critically important for patients who suffer because of these misconceptions and their family and friends make the life tougher by minimizing contact with them. This leads to more depressing situation for the patients. Oral fluid mode of HCV transmission is a controversy (Suzuki et al., 2005) but saliva has been found positive for HCV in many studies (Arend, 2000, Suzuki et al., 2005). That's why most of the subjects included in the study were also confused regarding salivary route of HCV transmission.

In our study medical student who represent well educated part of society and are at the start of their clinical rotation, were found to be not well equipped to face the challenge being unaware of the knowledge they were supposed to be. Previous studies conducted regarding awareness among medical students in Pakistan also showed their inadequate level of knowledge. A survey was conducted in Karachi and their study showed that medical students entering into the professional life did not have sound knowledge of diseases such as Hepatitis and AIDS and the students need more education and emphasize to take universal precautions (Anjum et al., 2005). Another survey conducted in 3 medical colleges i.e., Rawalpindi Medical College, Islamic International Medical College and Shifa Medical College showed important shortcomings in the knowledge, attitude and practice of the medical students regarding HBV and HCV marking importance of further education of the students (Wajiha Raza,

2008). In a survey conducted at 7 dental schools of Taiwan, more than half of the Taiwanese dental students who participated in the study were not familiar to HCV infection (Hu et al., 2004). Students from Japan and France have shown that the students belonging to these developed countries have misconceptions with respect to their knowledge regarding infectious diseases i.e., HBV, HCV and HIV (Anjum et al., 2005). In the present study, we also found that our medical students were not fully aware of HCV. Medical students are at higher risk of acquiring infections as they have direct contact with patients and they also deal with blood (transfusions), surgical instruments and injections. There is a need to start training regarding infectious diseases precautions in countries lacking safety equipment, safety instructions for infectious diseases and staff vaccination programmes (Anjum et al., 2005).

In our study, the knowledge of the subjects included in the study was not satisfactory keeping in view the high prevalence of HCV infection in Pakistan. Overall patients infected with HCV had very little knowledge regarding routes of HCV transmission as compared to healthy subjects and medical students. Level of awareness was also lower among females in all groups as compared to males.

Education regarding routes of HCV transmission among patients and their house hold contacts is critical to control HCV prevalence in general population. In adequate knowledge and spreading wrong information may result in unnecessary isolation of the chronically infected HCV patients leading to depression and social disgrace (Tiftikci et al., 2009). In Pakistan also, awareness programs are needed among general public to increase knowledge regarding HCV to make this society HCV

free for new generations and a comfortable place to live for healthy subjects as well as patients.

Risk Factor Exposure among Subjects with HCV Infection History

A number of risk factors are well known for transmitting HCV infection. Multiple risk factors can be found in a single person (Yee et al., 2001). In our study, we also found multiple risk factors for HCV infection in patients most prominent being unsafe medical practices i.e., surgical operation including dental surgery and injections. About 65% of the women among HCV infected females had undergone surgery during delivering their babies. It is very serious situation as the threat is not only to the mother but also to the new born if the disease is not timely diagnosed. One of the mother included in the study acquired HCV infection during delivering her first baby through surgery. Days before delivering her second baby she came to know about her HCV infection through medical reports of hospital. She again had to undergo surgery for delivering her second baby and the second baby was found to be HCV infected. So it is alarming situation in Pakistani hospitals. It has been reported that in Pakistan's highest populated province Punjab, the risk factors for HCV transmission include therapeutic injections (44.3%), dental surgery (16.6%) blood transfusion (10.7%) etc (Mujtaba et al., 2011). A study conducted in Turkey also found surgery as the most common factor for HCV transmission (Karaca et al., 2006). Another study conducted in Egypt also observed unsafe medical practices as most common factor for HCV transmission (Reker and Islam, 2014). Unsafe injections and syringe reuse is unnecessarily common practice in Pakistan. Unnecessary injections are given because

of the concept and demand among patients that medicines via injection are more effective as compared to oral route. (Altaf et al., 2004) . The second most frequent risk factor found in our study was body piercing which is very common in females especially earlobe piercing. None of males was found to have body piercing. In a study conducted in Brazil, one of the prominent risk factor for HCV was body piercing and women had more frequent body piercing as compared to males (Kvitko et al., 2013).

In our study we found a number of risk factors responsible for HCV infection but unsafe medical practices are posing a serious threat to the society. There is a need to increase awareness among people regarding these risk factors. As part of awareness program, the participants of the study were briefed about what is HCV infection, its routes of transmission, signs and symptoms, diagnostic tests, and treatment options.

Socio-Economic Status

According to the Economic Survey (2013-14), 60.19% of the population is living below poverty line based on $2 per day set according to international standards for middle-income countries. This estimate is according to World Bank's Poverty Head Count Analysis 2014. Poverty definition according to the Economic Survey of Pakistan is "a condition of person/community not having enough finances/resources and basics to maintain minimum life standard well-being that is acceptable in society". Poverty is deprivation in well-being with low incomes, inability to access basic needs and services for survival with self-esteem. Poverty can also assessed as minimum number of calories taken/ with minimum

income satisfying necessary needs of a person/ day because 60% of the poor people's budget spends on food etc (Kakakhel, 2014).

We found that significant percentage of subjects with HCV infection history had low education level and living below poverty line (mostly jobless and depending on other means/ family members for their living and treatment) as compared to the healthy educated subjects. Low socio-economic status was found to be associated with high risk of HCV infection and poor prognosis in patients infected with HCV in Denmark , United States of America, Puerto Rico, France and Norway (Omland et al., 2013). In our study, we also found the subjects infected with HCV have lower education, low income per month and low employment status suggesting the low socio-economic status as another risk factor. These results have implications clinically as well as with respect to public health. People with low socio-economic status should be target for future studies with respect to HCV infection screening and other diseases as not only HCV, other diseases are also associated with people with low socio-economic status (Omland et al., 2013).

Part II: Quantitative research

In Pakistan, about 6% of the population is victim of HCV with predominant genotype 3 (75–90% prevalence). Interferon alpha (IFN-α) plus ribavirin is widely used standard therapy for chronic HCV treatment all over the world but pegylated interferon along with Ribavirin has higher response rate than that of standard interferon. In Pakistan, standard interferon therapy is commonly given because of

economic reasons. Pakistan government is also providing only standard interferon through Prime Minister's initiative programme. But the actual response rate of the standard interferon is observed to be lower than previously reported (Ahmad et al., 2012). Identifying the factors responsible for non-responsiveness can be helpful in predicting the treatment response. Resistance to therapy can be result of different factors including viral factors, host factors and HCV proteins induced molecular mechanism that inhibits IFN signaling cascade (Asselah et al., 2010). Hence in the current study we also focused on HCV genotype 3 infected patients. The patients were given twice course of therapy (24 weeks duration each) because of their non-response to first course of therapy and still high viral load observed. Now the question arises what can be the factors responsible for this unresponsiveness.

Host factors also affect response to therapy e.g., age, gender, race/ ethnicity and clinical conditions like insulin resistance, diabetes, liver cirrhosis, steatosis, body mass index (BMI) (Asselah et al., 2010). Some studies state both male and female are almost equally affected by the HCV and its genotypes (Ali et al., 2011, Qureshi et al., 2009) whereas other studies suggest male sex association with high prevalence and poor response rate (Poynard et al., 1998). We have also observed more males infected by HCV genotype 3 and less responsive to interferon therapy.

The Age (less than 35 years) is also a predictive factor for HCV treatment response to standard interferon alpha along with ribavirin in genotype 3 infected HCV patients (Bhutta S, 2011). According to Ahmed et al. (2009), in case of interferon standard therapy, age < 40 years is best predictive factor for sustained virological

response (Ahmed et al., 2011). Same observation was reported by Idrees and Riazuddin (2009) in their study conducted on 400 naïve chronic HCV infected patients (Idrees and Riazuddin, 2009). Our results also showed that the responders have an average age of about 35 years who responded to first course of IFN treatment as compared to the non-responders with the mean age of about 42 years who were non-responders to twice course of interferon therapy.

Some studies state both male and female are almost equally affected by the HCV and its genotypes (Ali et al., 2011, Qureshi et al., 2009) whereas other studies suggest male sex association with high prevalence and poor response rate (Poynard et al., 1998). We have also observed more males infected by HCV genotype 3 and less responsive to interferon therapy.

BMI is another significant factor affecting SVR (Kim et al., 2012), our results showed that the normal controls as well as the responders have normal BMI as compared to the non-responders whose mean BMI falls in the overweight category hence increase in BMI is also associated with the failure to interferon therapy among HCV patients.

We found that ALT and Bilirubin were significantly higher in HCV patients non-responders to interferon treatment as compared to the responders and the normal subjects. Elevated levels have been observed in non-responders to interferon treatment (Abbasi A, 2013). Initially normalization of ALT level was used to evaluate the treatment efficacy. Previous studies showed that ALT was found to be normal in 50%

of the patients treated with interferon but when therapy stopped, half of these patients had again elevated levels of ALT (Tong et al., 1997, Davis et al., 1989, Marcellin et al., 1991, Causse et al., 1991). Thus normal ALT rate (sustained biochemical response) was between 13-30% after 6 months of post-treatment. Tong et al observed 19% of the patients had normal ALT levels (biochemical responders) after cessation of treatment but 40% of these biochemical responders were positive for HCV RNA (virological non-responders). Among virological responders (negative for HCV RNA) 20% of responders had elevated ALT levels. So ALT level correlation with treatment efficacy have limitations, hence viral agent measurement is more reasonable and practical to determine treatment efficacy (Tong et al., 1997). We also found some mixed figures, 16% of the patients non-responders to interferon have normal ALT levels, whereas 40% of the responders had slightly elevated ALT levels. In our study, the levels of bilirubin were found to be normal in all groups though significant difference was observed among the mean values of all the groups. Khan and Sarwar (2009) also found the normal levels of bilirubin among HCV infected patients subjected to interferon therapy (Khan and Sarwar, 2009).

Among the viral factors affecting treatment response, HCV genotype and baseline viral load are important predictors of response to IFN alpha treatment and its duration (Asselah et al., 2010). HCV patients have risk to develop other disorders that are costly in treatment with high morbidity rate e.g., cirrhosis and HCC (Kanwal et al., 2011). Among them, cirrhosis develops as result of prolonged HCV infection (Seeff et al., 1992). In USA, the prevalence of cirrhosis among HCV infected patients was

found to be 18.5% in 2006 (Kanwal et al., 2011). The studies conducted by Khan et al., 2009, HCV is the leading cause of cirrhosis as 53.6% of the liver cirrhosis patients had anti HCV antibodies in their sera (Khan et al., 2009). In our study, 36% of the non-responders were found to have cirrhosis.

IFNAR1 was detected in 23 out of 50 HCV patients resistant to interferon therapy showing interferon receptor expression, a significant factor, contributing to the resistance to the treatment. The mean age is younger in responders and normal as compared to the nonreponders. Liver histopathological grades, stages and scores were significantly different between nonresponders positive for intrahepatic IFNAR1 expression as compared to non-responders negative for IFANR1 expression. Out of 50 HCV patients non responders to standard IFN treatment, there were 18 patients with liver cirrhosis and were negative for intrahepatic IFNAR1 expression hence showing that the lack of expression may be the cause of advanced histopathological state of the liver and non-response to therapy. In addition to cirrhotic patients some of non-cirrhotic patients non-responder to IFN treatment were also not producing IFNAR1. Hence IFNAR1 is found to be a major factor affecting treatment response in HCV patients in our study and disease severity. Our findings were similar to Chandra et al., who presented evidence that ER stress (induced by interference in ER functions) induced by HCV and autophagy results in degradation of IFNAR1 and other factors. They found 50% reduction in expression of IFNAR1 in liver biopsies of HCV infected chronic liver disease and IFNAR1 expression was impaired in almost all patients with liver cirrhosis. Reduction in IFNAR1 expression can be due to suppression of innate

immune response and progression of liver disease. Immune response suppression leads to carcinogenesis and hence can be the reason why cirrhosis is a risk for development of HCC. They also found that IFN- λ receptor expression was not affected by HCV-derived ER stress and autophagy suggesting the IFN-λ treatment to overcome treatment resistance by HCV (Chandra et al., 2014). Our study is also supported by the studies conducted earlier (Salama H, 2007, Mathai et al., 1999, Morita et al., 1999, Massirer et al., 2004, Taylor et al., 2000, Gao et al., 2004). The importance of IFNAR1 can be judged by the findings of the study conducted by Datta et al., 2011 (Datta et al., 2011) that the full-length expression of IFNAR1 clone alone has the ability to restore defective signaling of Jak-Stat pathway, phosphorylation of Stat1-3, nuclear translocation and anti-HCV response in all cell lines resistant to IFN-α. The reason why 23 out of 50 HCV patients are positive for IFNAR1 mRNA expression but still resistant to interferon therapy is that there can be mutations in IFNAR1 sequence as has been observed in a study conducted by Naka et al 2005 (Naka et al., 2005a). Our studies show that lower expression of IFNAR1 is the reason responsible for interferon resistance in HCV patients and be considered a predictive factor for interferon resistance.

However apart from IFNAR1, we also analyzed the qualitative expression of IFNAR2 in liver tissues. Almost all the patients expressed IFNAR2 and STAT1. Our study was consistent with that conducted earlier where IFNAR2 was expressed by 90% of all the patients resistant to interferon therapy and hence is not major cause of resistance to therapy in HCV patients (Gulshan A. Trali et al., 2014). Similarly

STAT1 is also expressed by responders as well responders and hence does not seem to be major cause of resistance to IFN therapy in HCV patients. These findings are similar to conducted by EL-Saadany but he found the main factor is STAT1 activation i.e., phosphorylation and hence STAT1 activation is important to conduct anti-viral actions and can be the treatment response predictor (El-Saadany et al., 2013) . According to Helbig et al, HCV genomic replicon blocked the IFN-α-induced STAT activation in hepatic cell line (El-Saadany et al., 2013, Helbig et al., 2008).

There can be different elements associated with interferon resistance in HCV patients like viral and host factors and the HCV proteins triggered molecular mechanisms that play important role by inhibiting IFN stimulated signaling pathway (Asselah et al., 2010). Studies have suggested that HCV proteins are important HCV to evade from IFN response (El-Saadany et al., 2013). Low quality of interferon used for HCV treatment that compromises the costs can induce false/ pseudo interferon resistance.

CONCLUSION

IFNAR1 can be used as a predictor factor of response to IFN standard therapy as well as disease severity. The patients expressing IFNAR1 have better chances to respond to treatment. But in case of resistance to IFN-alpha, the alternative IFN- λ treatment can be a good option. Our study also concludes that surgery/ medical malpractice is the major risk factor in our population. Besides the exposure to risk factors, awareness regarding HCV and low socioeconomic status also seem to be risk

factors for HCV in our study. There is inadequate awareness regarding HCV in Pakistani population. Measures should be taken to educate people regarding possible risks of acquiring HCV on urgent basis. It is time to educate the clinical students, patients, normal population regarding basic facts and figures about HCV so that they can better perform their role in preventing the transmission of this deadly disease. Policies should be approved at governmental level for timely diagnosis and prevention of this devastating virus. Socio-economic status should be considered another risk factor for acquiring HCV infection and future studies should target the low socio-economic groups for research and other policies making purposes.

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APPENDICES

APPENDIX I: QUESTIONNAIRE

Demographic data

1. Name: _____ 2. Age:_____

3: Gender:_____ 4: Marital status: _____

Socio-Economic Status:

1. Education:
2. Employment/occupation:_____
3. Income:_____

Risk factors Exposed:

Risk Factor	Tick please
1. Blood transfusion	
2. Dental surgery(Classified)	
3. Dental surgery (Non-classified)	
4. Contact with blood/needle	
5. Sharing syringes	
6. Sharing tooth brush	
7. Sharing razor	
8. IDU	
9. Barber shave	
10. Surgical operation	
11. Blood loss due to accident	

12. History of cuts	
13. Ever admitted hospitals	
14. Body piercing	
15. History of injection	
16. Kidney dialysis	

Knowledge regarding HCV transmission sources

Source of transmission	Agree	Disagree	Have no idea
1. Blood/ blood products			
2. Injection drug users			
3. Sharing utensils			
4. Sharing tooth brushes			
5. Sharing of blade			
6. Sexual contact			
7. Coughing and sneezing			
8. Casual contact			
9. Saliva			
10. Syringes			
11. HCV Infected Mother's transferred the disease to baby			

APPENDIX II

AGAROSE GEL

	1.0 %	**1.5 %**
Agarose	1.0 g	1.5 g
1 % TAE buffer (pH 8.0)	100 ml	100 ml

Agarose gel was dissolved by microwaving for 2 minutes, cooled to 50 ˚C and poured into the gel tray.

50 X TRIS- ACETATE- EDTA (TAE) BUFFER

Dissolve 242 gms of tris base, 57.1 ml of glacial acetic acid and mix 100 ml of 0.5M EDTA (pH 8) in 500 ml of distilled water making the volume up to 1 liter and 1X concentration was used.

ETHIDIUM BROMIDE

Ethidium bromide (1 percent)	10 µl
Distilled water	100 ml

Dissolve 1gm of ethidium bromide in 100ml of water and wrap the container in aluminum foil/ store in dark bottle. Store indefinitely at room temperature.

AWARDS

- NUST Indigenous Mega S & T merit Scholarship

- Won the IUIS - Gates Foundation Travel Grants for Young Immunologists from Developing Countries to attend 15th International Congress of Immunology – ICI held at Milan, Italy from 22nd – 27th August, 2013

- Won the Junior Research Scholarship Award to attend the Viral Hepatitis Congress 2012 held at Johann Wolfgang Goethe University, Frankfurt, Germany from 7th – 9th September 2012.

SELECTED INTERANTIONAL PUBLICATIONS

- **Q. Bashir**., A. Rashid., A. k. Naveed., S. A. Khan, A. Younis., S. Razak. Interferon Signaling Pathway: Intrahepatic Expression Analysis of STAT1 in HCV Patients Resistant to Interferon Therapy. Khyber Medical University Journal, 2013, Vol. 5 No. 3.

- **Q. Bashir**., A. Rashid., S. Razak. Hepatitis C Virus (HCV) Awareness among Medical Students in Rawalpindi. Pakistan Armed Forces Medical Journal (2015) (Accepted).

- **Q. Bashir**., A. Rashid., S. Razzak. Intrahepatic Expression Analysis of Interferon Alpha Receptor 1 in Hepatitis C Patients Resistant to Interferon Therapy. (In process)

- **Q. Bashir.,** A. Rashid., S. Razzak. Risk Factors for Hepatitis C: Low Socio-Economic Status and Hepatitis C Awareness Level (In process)

- G. A. Trali., A. Rashid., A. k. Naveed., **Q. Bashir.,** A. Majeed., S. A. Khan, S. Razzak. Detection of Interferon Alpha Receptor 2 in Interferon Resistant HCV Patients. Hepato-gastroenterology, 131, 752-54.

SELECTED INTERNATIONAL PRESENTATIONS

- **Bashir, Q.** Interferon Alpha Recepetor and Resistance to Interferon Therapy in HCV Patients. Oral presentation to the scientists at Boditech Med Inc. Chuncheon si, Gang-won-do, South Korea on 14th October 2013.

- **Bashir, Q.,** Rashid, A., Naveed, A.K., Khan, R. S. A., Razak, S. Interferon signaling pathway: Intrahepatic expression of STAT 1 and interferon resistance in HCV patients. 15th International Congress of Immunology – ICI held at Milan, Italy from 22nd – 27th August, 2013.

- **Bashir, Q.,** Rashid, A., Naveed, A.K., Khan, R.S.A., Trali, G.A., Gul, A. IFNAR2 Intrahepatic Expression of Interferon Alpha Receptor 1 (IFNAR1) in Hepatitis C Patients Resistant to Interferon Therapy. Viral Hepatitis Congress 2012 held at Johann Wolfgang Goethe University, Frankfurt, Germany from 7th – 9th September 2012. Journal of Viral Hepatitis, 2012, 19 (Suppl. 3), 5–30.

- **Q, Bashir,** G, A, Trali., A, K, Naveed., A, Rashid., R, S, A, Khan., S, Razak. Intrahepatic Expression Detection of Interferon Alpha Receptor 2 in Interferon Respondent HCV Patients. Oral presentation at National Symposium on "Biochemical and Molecular Basis of Human Diseases" held at University of Gujrat, Gujrat, Pakistan from 17th – 18th May 2012.

Centre for Research in Experimental and Applied Medicine (CREAM)
Army Medical College,
Abid Majid Road,
Rawalpindi, Pakistan
Tel: 051-561-31457, Ext-281
051-5584352

No. 02/CREAM-A-4

01 Feb 2013

CERTIFICATE

The Ethical Committee of Centre for Research in Experimental and Applied Medicine (CREAM), Army Medical College has evaluated the ethical aspect of the research proposal titled "Liver biopsies collection from interferon resistant HCV patients and responders to interferon therapy/normal subjects " and accorded approval.

Brig
President Ethical Review Committee
(Prof M. Mazhar Hussain)

To: Biochemistry Dept

Info: Dr. Qudsia Bashir

CERTIFICATE

IUIS - Bill and Melinda GATES Foundation Travel Awards for Young Immunologists from Developing Countries

Awardee's name: **Dr. Qudsia BASHIR (Pakistan)**

To Whom It May Concern

This is to certify that you are one of the laureates of the **IUIS-GATES Foundation Travel Grants for Young Immunologists from Developing Countries**. We received over 750 applications from around the world, and we granted 36 awards, including the award to you. Awardees were selected by an independent international scientific jury based upon the quality of the abstract submitted for communication at the 15th International Congress in Immunology held in Milan, August 22-27, 2013.

Congratulations on your award!

Sincerely,

Jean-Loup Romet-Lemonne, M.D.
IUIS Program Director
Immunology without Borders
175 Varick Street
New York, NY 10014
www.iuisonline.org

THE VIRAL HEPATITIS CONGRESS

Certificate of Attendance

This is to certify that

Qudsia Bashir

attended the

VIRAL HEPATITIS CONGRESS

7–9 September 2012
at the
J.W. Goethe University
Frankfurt, Germany

Professor Ira Jacobson
Co-Chair

Professor Stefan Zeuzem
Co-Chair

'The Viral Hepatitis Congress' is accredited by the European Accreditation Council for Continuing Medical Education (EACCME) to provide the following CME activity for medical specialists. The EACCME is an institution of the European Union of Medical Specialists (UEMS), www.uems.net.

'The Viral Hepatitis Congress' is designated for a maximum of (or 'for up to') 13 hours of European external CME credits. Each medical specialist should claim only those hours of credit that he/she actually spent in the educational activity. Through an agreement between the European Union of Medical Specialists and the American Medical Association, physicians may convert EACCME credits to an equivalent number of AMA PRA Category 1 Credits™. Information on the process to convert EACCME credit to AMA credit can be found at www.ama-assn.org/go/internationalcme.

Live educational activities, occurring outside of Canada, recognized by the UEMS-EACCME for ECMEC credits are deemed to be Accredited Group Learning Activities (Section 1) as defined by the Maintenance of Certification Program of The Royal College of Physicians and Surgeons of Canada.

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October 15, 2013

To whom it may concern,

Dr. Qudsia Bashir has presented her work entitled "Interferon Alpha Receptor and Resistance to Interferon Therapy in HCV Patients" to our scientists on October 14th 2013.

Sincerely yours,

Soon-cheol Hong, Ph.D.

Director of Academics and Information

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National Symposium on

"Biochemical and Molecular Basis of Human Diseases"

May 17-18, 2012

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

A JOINT VENTURE OF NAWAZ SHARIF MEDICAL COLLEGE (NSMC) & INSTITUTE OF CHEMICAL AND BIOLOGICAL SCIENCES (ICBS)

UNIVERSITY OF GUJRAT, GUJRAT, PAKISTAN

Certificate of Participation

This is Certify that Ms. Qudsia Bahir has presented following research work as Oral Presentation in National Symposium on "Biochemical and Molecular Basis of Human Diseases

Intrahepatic Expression Detection of Interferon Alpha Receptor 2 in Interferon Respondent HCV Patients

Qudsia Bashir, Gulshan Ara Trali, Abdul Khaliq Naveed, Amir Rashid, Rao Saad Ali Khan, Suhail Razak

Dr. Sajid Mehmood
Head of Department/Secretary BMBHD