Studies on hepatitis C virus non-structural gene 3 mutations and HCV host pathogenesis

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CERTIFICATE FROM THE SUPERVISOR

This is to certify that the thesis entitled **"Studies on hepatitis C virus non-structural gene 3 mutations and HCV host pathogenesis"** submitted by Sri Supradip Dutta who got his name registered on **11th September, 2019** for the award of Ph. D. (Science) Degree of Jadavpur University, is absolutely based upon his own work under the supervision of **Dr. Provash Chandra Sadhukhan** and that neither this thesis nor any part of it has been submitted for either any degree/diploma or any other academic award anywhere before.

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Declaration

The research work embodied in this thesis entitled "**Studies on hepatitis C virus non-structural gene 3 mutations and HCV host pathogenesis**" being submitted to Jadavpur University, Kolkata has been carried out at ICMR-National Institute of Cholera and Enteric Diseases, Beleghata, Kolkata under the supervision of Dr. Provash Chandra Sadhukhan, Virus laboratory, ICMR-National Institute of Cholera and Enteric Diseases. The work is original and has not been submitted in part or in full, for any degree or diploma to this or any other university.

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Dedication:

Dedicated to all who fight against the deadly virus

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After a long time and years of research, thinking, experimenting, and disappointments, this thesis is finally complete. While writing these final words, I realize that many people have helped, supported, or contributed to this thesis and deserve acknowledgement.

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List of Abbreviations

AASLD	American Association for the Study of Liver Diseases.
ADCC	Antigen Dependent Cellular Cytotoxicity
AIDS	Acquired Immunodeficiency syndrome
ALT	Alanine transaminase
AMP	Adenosine monophosphate
AMV	Avian Myeloblastosis Virus
AP-1	Activator protein 1
APASL	Asian Pacific Association For Study Of Liver
APC	Antigen Presenting Cells
ASPP2	Apoptosis stimulating of p53 protein 2
AST	Aspartate Aminotransferase
ATP	Adenosine triphosphate
BALB/c	Bagg Albino mice
BCE	B-cell Epitopes
BEAST	Bayesian Evolutionary Analysis Sampling Trees
BLAST	Basic Local Alignment Search Tool
BV-BRC	Bacterial and Viral Bioinformatics Resource Center
CCR	Chemokine receptor
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
CD81	Cluster of differentiation 81
CDS	Coding sequence
CED	Conformation Epitope Database
CFDA	Carboxyfluorescein diacetate
CFSE	Carboxyfluorescein succinimidyl ester
CHAD3	Chimpanzee adenovirus type 3
CI	Confidence Interval
CKD	Chronic Kideney Disease
CLD	Chronic Liver Disease
CO2	Carbon dioxide
COX	Cyclooxygenase

CRF	Case Report Form
CTL	Cytotoxic T cell
CXCL	chemokine (C-X-C motif) ligand 1
D1	domain 1
D2	Domain2
D3	Domain 3
DAA	Direct Acting Antiviral
DCLD	Decompensated Chronic Liver Disease
DCV	Daclatasvir
DDI	Drug-drug Interaction
DMSO	Dimethyl sulfoxide
DNA	Deoxy Ribonucleic Acid
DOPPS	Dialysis Outcome and Practice Pattern Study
DTT	Dithiothreitol
E1	Envelop 1
E2	Envelop 2
EASL	European Association for the Study of the Liver,
EDTA	Ethylenediamine tetraacetic acid
EEA	European Economic Area
ELISA	Enzyme-linked Immunosorbent Assay
ELISPOT	Enzyme-linked immunosorbent spot
EMBO	European Molecular Biology Organization
EMRO	WHO Regional Office for the Eastern Mediterranean
ER	Endoplasmic Reticulam
ERK	Extracellular signal-regulated kinase
ESRD	End-Stage Renal Disease
ESS	Effective Sampling Sizes
EU	European Union
FACS	Fluorescence-activated cell sorting
FAM	Fluorescein amidites
FBS	Fetal bovine serum
FDA	Food and Drug Administration
GFR	Glomerular filtration rate

GT	Genotype
GTP	Guanosine triphosphate
HBA1	Hemoglobin subunit alpha 1
HBA2	Hemoglobin subunit alpha 2
HBB	haemoglobin beta gene
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
HIV	Human immunodeficiency virus
HKY model	Hasegawa-Kishino-Yano model
HLA	Human leukocyte antigens
HPLC	High-performance liquid chromatography
HRP	Horseradish peroxidase
HVR	Hypervariable region
IDSA	Infectious Diseases Society of America
IDU	Intravenous Drug user
IEC	Institutional Ethics Committee
IEDB	Immune Epitope Database
IFN	Interferon
IL	Interleukin
IL10	Interleukin 10
IL4	Interleukin 4
INASL	Indian National Association for Study of the Liver
IRES	Internal Ribosome Entry Site
IU	International Unit
JNK	Jun N-terminal kinase
LDL	Low Density Lipoprotein
LDV	Ledipasvir
LSM	Leucocyte Separation Media
MAPK	Mitogen-activated protein kinase
MAV	Mitochondrial antiviral-signaling protein
MCMC	Markov Chain Monte Carlo

MEGA	Molecular Evolutionary Genetics Analysis
METAVIR	Meta-analyses of histological data in viral hepatitis
MFI	Mean fluorescence intensity
MG	Membranous glomerulopathy
MHC	Major Histocompatibility
f-MOC	Fluorenylmethoxycarbonyl protecting group
MPGN	Membranoproliferative glomerulonephritis
MRR	Mortality rate ratio
MVA	Modified vaccinia
NACO	National AIDS Control Organisation
NAT	Nucleic Acid Testing
NBM	Nucleotide binding motif
NCBI	National Center for Biotechnology Information
NCR	Non-coding region
NGO	Non-Governmental Organisation
NIAID	National Institute of Allergy and Infectious Diseases
NICED	National Institute of Cholera and Enteric Diseases
NIH	National Institutes of Health
NK cells	Natural Killer cells
NLS	Nuclear localization signal
NMR	Nuclear Magnetic Resonance
NS region	Non-structural region
NS1	Non-structural region 1
NS2	Non-structural region 2
NS3	Non-structural region 3
NS3INF	NS3 inner forward
NS3INR	NS3 inner reverse
NS3OUF	NS3 outer forward
NS3OUR	NS3 outer reverse
NTD	N-terminal domain
NTP	nucleoside triphosphate
NVHCP	National Viral Hepatitis Control Program
NaOAc	Sodium Acetate

OD	Optical density
ORF	Open Reading Frame
PAGE	Polyacrylamide gel electrophoresis
PAMP	pathogen-associated molecular patterns
PBMC	Peripheral Blood Mono nuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PDB	Protein Data Bank
PEG	Poly Ethylene glycol
PHA	Phytohemaglutinine
PI3	Phosphoinositide (PI) 3-kinases
PPAR	Peroxisome proliferator-activated receptor
PWID	People Who Inject Drugs
QRT	Quantitive Real time
RAS	Resistance Associated Substitution
	Research Collaboratory for Structural Bioinformatics
RCSB	Protein Data Bank
RDRP	RNA-dependent RNA polymerase
RIB	Ribavirin
RIG-1	Retinoic acid-inducible gene I
RMSD	Root Mean Square Deviation
RNA	Ribo-nucleic Acid
RO	replication organelles
ROS	Reactive Oxygen Species
RPMI medium	Roswell Park Memorial Institute (RPMI) medium
RT	Reverse Transcriptase
RV	Reverse Vaccinology
SARS	Severe Acute Respiratory Syndrome
SF	Super families
SL	Stem loop
SOF	Sofosbuvir
SVR	sustain viral response
TAMRA	Tetramethyl-rhodamine

TB	Tuberculosis
TCE	T-cell receptors
TLR	Toll-like Receptor
TM1	Trans Membrane helix 1
TM2	Trans Membrane helix 2
TMD	c-terminal domain
TN model	Tamura-Nei model
TNF	Tumour Necrosis factor
Q-TOF	Quadrupole Time-of-Flight (QTOF) Mass Spectrometry
TTI	Transfusion Transmitted Infection
UI	Uncertainity Index
UK	United Kingdom
USA	United States Of America
UTR	Untranslated Region
VEL	Velpatasvir
VLP	Virus like particles
WHO	World Health Organization

Summary

The Hepatitis C Virus (HCV) is a major public health problem worldwide. It causes HCV-mediated chronic liver disease and end-stage liver diseases like hepatocellular carcinoma. Approximately 58 million people are suffering from HCV infection throughout the world. In India, it is estimated that 6-12 million people are HCV-infected which accounts for a large portion of global HCV prevalence. Transmission of HCV is mainly due to blood-to-blood contact like injecting drugs, poor blood transfusion methods, and unsafe clinical practices. HCV infection is often silent in the early days of infection but it can become severe and fatal after several years of infections. Thus, it is also known as "Silent Killer." World Health Organization has taken the initiative to eradicate HCV infection by 2030 but no vaccine for this virus is available till now. The high error rate of RNA-dependent RNA polymerase (RdRp) of HCV gives rise to 8 genotypes and more than 86 subtypes. These genotypes also vary depending on the host's (human) race, sex, and geography of distribution. In Europe and North America, HCV genotype 1 is prevalent, whereas in Asia genotype 3 is prevalent, and in Africa genotype 4 is prevalent. HCV genotype 3 is mostly prevalent in India although other unusual genotypes and subtypes (1c, 4a, 6h, 6n, 6k and 8) can also be found. The genotype distribution pattern of HCV in India is still hazy. The DAAs therapy guideline is also dependent on HCV genotype to some extent. The high genetic variation of HCV is the main challenge for vaccine development against HCV. Till the last decade, Pegylated Interferon (PEG-IFN) and Ribavirin-based treatments were the only options for the treatment of this virus. However, after 2015 direct-acting antivirals (DAAs) for HCV came to market which helped to restrict HCV infection drastically. Still, there are several limitations to using DAAs for all and the new DAA-resistant HCV cases indicate that the vaccine is a compressing need of the hour.

HCV non-structural protein 3 (NS3) is a bi-functional enzyme (protease and helicase) which plays a major role in viral replication. It has long been targeted for therapeutic intervention of HCV. NS3 protein-specific T-cell response was found to be associated with viral clearance. Mutations of NS3 protein are also said to have a connection with HCV pathogenesis and cancer. The major focus of this thesis is to spotlight the epidemiology and genotype distribution of HCV, virus-mediated pathogenesis, mutational analysis of NS3 protein and evaluate MHC-I and MHC-II epitopes on the NS3 gene of HCV genotype 3 using in-silico and *In-vitro* approaches.

It was found that, out of 661 anti-HCV-positive samples examined, 535 samples (80.39%, 535/661) belonged to the high-risk groups and the remaining 126 were from the general population with Chronic Liver Disease (CLD). Out of 661 samples, 403 samples (60.96%) were RNA-positive. The highest viremia was observed in the People Who Inject Drugs (PWIDs) population (70.70%), followed by haemophilia (62.50%) and thalassemia (65.21%). Genotype distribution of HCV in different population groups revealed an interesting finding. HCV subtype 3a (76.69%) is mostly prevalent in the thalassemia population, whereas subtype 1c (67.95%) was predominant in the CKD population. On the other hand, in the case of general population subtypes 3a (36.71%) and 3b (34.18%) were found to be almost equally prevalent. Overall, Genotype 3 was found to be the most prevalent genotype in this region.

The phylogeographic study reveals that subtype 3a strains were related to Sri Lanka, Russia, Pakistan, Myanmar, and Thailand. Whereas, subtype 1c showed a resemblance with isolates from Indonesia, China, Cameroon, and Myanmar. HCV subtype 3b isolates found in this region shared common ancestors with China, Myanmar, Vietnam, Thailand, and other Southeast Asian isolates. Subtype 1b shared similar ancestors with Japan, Myanmar, and Thailand isolates. Subtype 1a had links with other nations' isolates, including China, Venezuela, Germany, and Iran. Subtype 4a very possibly, drifted from Saudi Arab.

HCV also showed disease complexities in high-risk groups like thalassemia and chronic kidney disease (CKD). An NS3 mutation N224T might relate to decompensated liver disease progression. HCV also showed to augment ESRD in CKD patients and further liver inflammation in thalassemia patients.

In this study, The MHC-I and MHC-II epitopes were found utilising the IEDB server. Antigenicity, non-allergy, non-toxicity, non-human homology, and other important factors were utilised to choose anticipated epitopes. Conservancy analysis was also performed on filtered epitopes. Docking was carried out on highly conserved epitopes utilising several MHC-I and MHC-II allele structures obtained from PDB. The top-scoring epitopes from docking data were synthesised and tested in *In-vitro* utilising CFDA-SE cell proliferation and IFN-γ gamma assays.

The top-scoring MHC-I and II epitopes showed encouraging outcomes in T cell proliferation and IFN-γ response in *In-vitro*.

This thesis comprised HCV epidemiology, pathogenesis, genotype distribution and mutational changes within the NS3 protein across the different genotypes. Epitopes predicted and validated in this thesis may be further utilised to produce vaccination against HCV in the future.

Chapter 1

1 Introduction:

Hepatitis C Virus (HCV) is one of the leading causes of liver cirrhosis and hepatocellular carcinoma (HCC) around the World. HCV is often unrecognised in the preliminary stages and creates a difficult situation for clinicians as well as patients. An estimated 58 million people have been suffering from the HCV virus infection globally, with an estimated new infection rate of about 1.5 million every year (1). HCV is a blood-borne pathogen transmitted through cross-contamination of blood and body fluids. People who inject drugs (PWIDs), patients undergoing haemodialysis or having chronic kidney disease (CKD), and patients with haemophilia or thalassemia are therefore at high risk for HCV infection (2–4). HCV can cause both acute and chronic infections. It is said that 15-40% of the infected population can clear the virus spontaneously, and the rest 60- 80% of the population become chronically infected which eventually leads to liver cirrhosis and hepatocellular carcinoma (HCC) within 20-30 years (5). The rate of development of cirrhosis also depends on age, sex, gender, and other immune responses (6,7). The mutation rate of the HCV virus is very high which leads to 8 genotypes and more than 86 subtypes (8,9). The versatility of the HCV virus is the major hindrance towards vaccine development.

1.1 Global scenario of HCV prevalence:

HCV is spread all over the World. The highest viraemic HCV prevalence was seen in Central Asia (3.6%, 95% UI: 2.8-3.9), in contrast to Western Europe or Central and Andean Latin America (0.5%, 95% UI: 0.4-0.5/0.3-0.6) where viraemic HCV prevalence found to be very low. Sub-Saharan Africa also showed a relatively high prevalence of viraemic HCV (10). In

the United States, about 2.7-3.9 million people have been suffering from HCV and 75% of them are unaware of this (11). In the UK about 81 thousand people have been suffering from chronic HCV (12). In Australia, approximately 2 million people have been suffering from HCV infection (13,14). Most HCV-prevalent countries in the World may belong to Central Sub-Saharan African countries like Cameroon (13.8%), Burundi (11.3%), and Gabon (9.2%) (15). Among Eastern European countries, Russia has the highest HCV prevalence (4.1%) (16). Maybe this is because Russia shares boundaries with many Asian countries and also some part of Russia lies within Asia. Eastern Asian countries like Japan and Singapore have relatively low HCV prevalence (0.5%). Whereas, 94.6 million people were infected with HCV in Southeast Asian countries (17). Unfortunately, till now there are many countries, which did not have robust studies which give a clear insight into HCV prevalence.

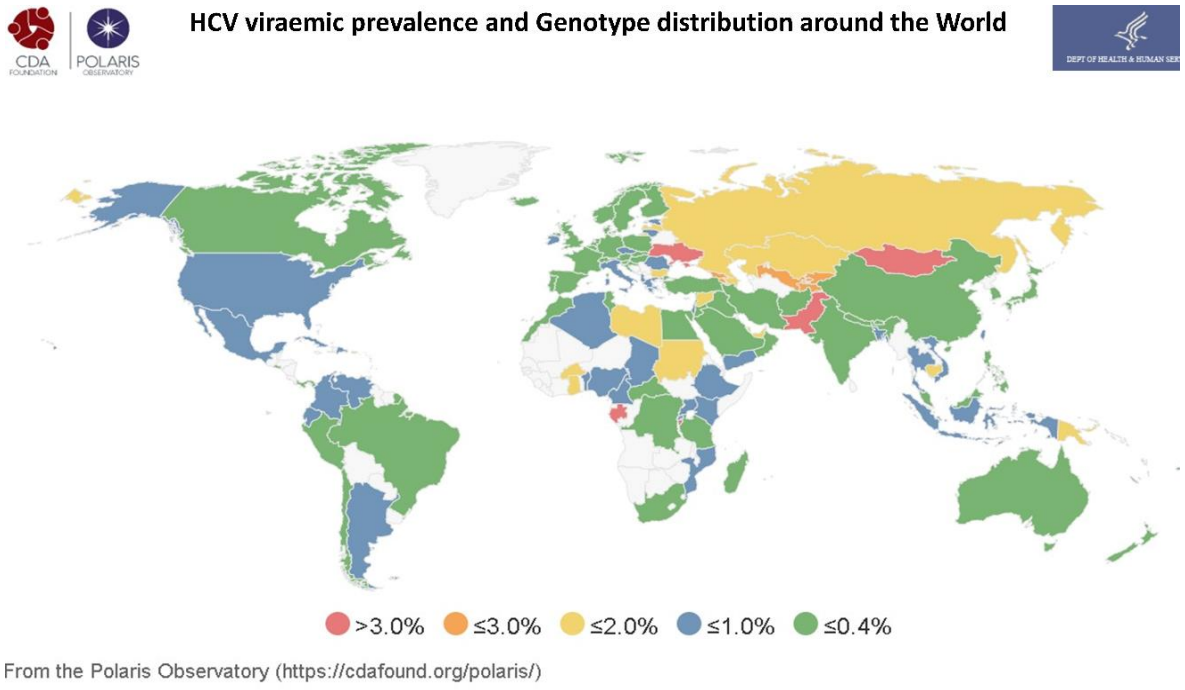

Figure 1: HCV prevalence around the World.

1.2 Genotype distribution of HCV around the World:

Genotype distribution of the HCV virus depends on geographic factors (18). Genotypes 1a and 1b can be found all over the world. Genotype 1 is the most prevalent genotype around the world followed by genotype 3(19). Genotypes 1 and 2 are mostly prevalent in the USA, Europe, and Australia. Whereas genotype 3 is prevalent in central Asia, HCV genotype 4 is mostly found in the Middle East and Africa specifically Egypt, genotype 5 is most abundant in South Africa and genotype 6 is most common in Southeast Asian regions (20,21). Genotype 7 was identified in Congo, Central Africa (22). Recently, Genotype 8 was identified in Punjab, India (8).

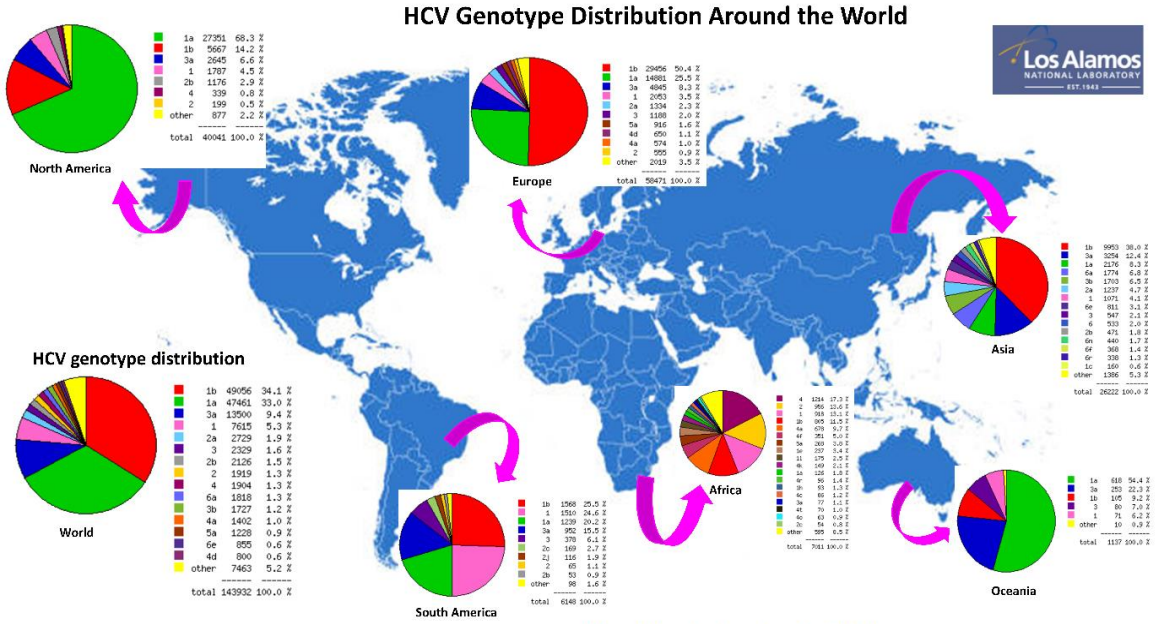

Figure 2: HCV genotype distribution around the world.

1.3 Indian scenario concerning HCV infection:

HCV prevalence in India lies between 0.5% to 1.5%. Northeastern states, Punjab and its adjacent areas are considered hotspots for HCV infection (23). It is estimated that 6-12 million people in India have been suffering from HCV(24) which is a major portion of global HCV infection. As per studies, anti-HCV prevalence was 0.29-1.85% in Northern states of India,

0.08-1.4% in South-Indian states, 0.27-1.17% in Northeastern states of India and 0.31-1.09% in Eastern-India. Whereas, Western states of India show a relatively low (0-0.9%) anti-HCV prevalence (23). India needs to become more cautious with its HCV control plan because it bears nearly 10 million thalassaemic individuals and 15 million sickle cell disease(25). Other than this India also accounts for near about 17 million dialysis patients (26) and roughly 17 million intravenous drug users (People Who Inject Drugs/PWIDs). These population groups are highly vulnerable towards HCV infection. Studies reported high HCV prevalence among these groups (4,27–29). Region-wise HCV prevalence remains unclear (10) and the risk groups may play a role in this controversy. Genotype 3 is the most common in India followed by genotypes 1 and 4 (30). However, other genotypes are also can be found regional basis. For example, HCV genotype 6 can be found among the PWIDs population in the border states of Manipur, part of North-East India (31). Genotype 2 can be found in Karnataka, a south Indian state (32). Recombination of genotypes also has been reported in India (30,33). Recently a new genotype 8 has been found in Punjab, Northern India (8). As per subtype-based HCV prevalence, genotype 3a is the most prevalent one (34). Unusual subtypes like 3g and 3i also

Figure 3: Genotype distribution of HCV in India.

can be found in small numbers among thalassemia (4). All these observations can deduce detailed epidemiological studies of HCV with genotype and subtype distribution in the Indian situation are needed.

1.4 Route of transmission:

The main route of HCV transmission occurs via blood-to-blood contact. HCV is therefore called a blood-borne virus. HCV is generally spread through contaminated needle sharing, accidental needle pricks, unscreened blood or blood product transfusion, contaminated medical devices, tattooing, and so on. HCV-infected people serve as a reservoir for new infections (35,36). HCV can also spread through male homosexual practices (37). HCV can also spread sexually in low prevalence (~1.2%) (38), but the chance increases with HIV co-infection (39). Modes of transmission of HCV are different among developed countries and developing countries. In developed countries, injecting drugs and sexual transmission are more common in HCV infection, whereas, in developing countries, it has been seen that HCV has spread through unsafe clinical procedures or contaminated medical equipment (40).

1.5 Stages of Infection and severity of disease:

HCV may often remain silent in the initial days but it gets worse with time. Within 20-30 years that infection leads to liver cirrhosis (41,42). Generally, there are 4 stages of HCV-mediated liver cirrhosis. They are-

- Stage 1: mild fibrosis of the liver without wall damage
- Stage 2: moderate fibrosis of the liver with small scares
- Stage 3: fibrosis had been spread all over the liver with more scares.
- Stage 4: severe liver damage or cirrhosis.

These stages depend on the METAVIR score (meta-analyses of histological data in viral hepatitis) (43,44). Rapid progression of HCV-mediated liver fibrosis depends on several factors like Age (>40 years), Daily alcohol intake(>50g), Gender (male) and other factors (43,45–47). When cirrhosis is established, hepatic decompensation and HCC development start gradually at a rate of 1-4% per annum (5), with a mortality rate ratio (MRR) of -1.55[95% CI:1.28-1.86] (48). The damaged hepatic function may revert back and lifestyle may improve after antiviral therapy but not for all patients and liver transplantation often be the only way (49). Therefore, HCV infection is deadly, silent, and expensive to manage.

1.6 Treatment and Management of HCV:

Antiviral Ribavirin and pegylated interferon alpha/beta (IFN-α/IFN-β) were the only treatment option for HCV over the last decade (50). However, the efficacy of that treatment was not satisfactory (51). There were many side effects of Ribavirin+peg-interferon therapy (52,53). To overcome these obstacles, in 2011, the first Directly Acting Antivirals (DAAs) against HCV was introduced. Which was an NS3 protease inhibitor. That developed the efficacy of the treatment but side effects indicated that more safer drugs should be needed. In 2013, NS5b inhibitor sofosbuvir was introduced, which finally showed good efficacy with lower side effects. These observations eventually led to FDA approval of the first DAA regimen with sofosbuvir/ledipasvir or sofosbuvir/simeprevir in 2014 (54). These drugs showed much lower side effects and improved efficacy. For all HCV genotypes other than Genotype-3, the Asian-Pacific Association for the Study of the Liver (APASL, 2016) advised using sofosbuvir (SOF) in combination with ledipasvir (LDV), and sofosbuvir (SOF) in combination with daclatasvir (DCV) for genotype-3 (55). The American Association for the Study of Liver Diseases (AASLD, 2019) and the Infectious Diseases Society of America (IDSA) have published guidelines for the HCV pan-genotype-based treatment (56). India first launched the National Viral Hepatitis Control Program (NVHCP) on 24th February 2019 based on “National

guidelines for diagnosis and management of Viral hepatitis" (57). Which is as follows for HCV management and treatment-

1.6.1 Diagnosis:

Serological testing for HCV antibodies is used to screen for HCV infection. A Nucleic Acid Test (NAT) for HCV RNA is required to confirm chronic HCV infection if the result is positive. It is crucial to test for HIV and HBV in addition to HCV and to consider the likelihood that people with HCV may also be infected with other blood-borne viruses. Several high-risk populations, such as HIV patients, convicts, and people who inject drugs (PWIDs), should also be tested for other illnesses such as tuberculosis (TB). HCV RNA must be done before treatment. PWIDs, men who have sex with men, female sex workers, people who live with blood transfusion (haemophilia, thalassemia) and dialysis, people with HIV, and inmates of prisons- these groups must be focused for testing. HCV genotyping is also a must.

1.6.2 Other assessment:

A detailed history of alcohol consumption must be taken from the patients as well as other medications patients might be using also have to be collected before treatment. Alcohol consumption must be reduced by patient counselling before treatment. The degree of fibrosis must be considered before the treatment. selection of treatment regimens must be decided based on the presence or absence of cirrhosis. Other than that diabetes, thyroiditis, cryoglobulinemia and other risk factors should be considered before starting DAAs treatment.

1.6.3 Treatment regime:

Patients with cirrhosis must be treated with Sofosbuvir (SOF)+ Velpatasvir (VEL) for 12 weeks. Whereas, patients who do not have cirrhosis must be treated with SOF+DCV for 12

weeks. After 12 weeks again RNA testing must be done. If HCV RNA is further detected after treatment, then the patients must move to a new regime or else treatment gets completed. But if that patient has decompensated cirrhosis, Ribavirin (RIB)+ SOF +VEL should be recommended for 12 weeks, if that decompensated patient is not tolerated with Ribavirin, then SOF+VEL is recommended with a duration of 24 weeks. Patients who cannot clear the virus after 12 weeks of the above-mentioned regimes must follow the SOF+VEL+RIB regime for another 24 weeks. Following up on patients must be done carefully throughout the treatment. If a patient can clear the infection within the first 12 weeks of treatment, then it can be said that the patient has achieved a Sustained Viral Response-12 (SVR-12). If the patient cannot achieve SVR-12 then that patient also must be followed until he can clear the virus.

1.6.4 Other preventive management:

NVHCP also recommended several measures to prevent HCV infection and harm reduction for risk groups of HCVs. those are-

- HCV ELISA test must be done before storing blood in blood banks to ensure safe blood transfusions.
- HCV Screening of patients before any surgical procedure to prevent nosocomial spread of HCV infection.
- HCV risk group populations like Thalassemia, Chronic Kidney Disease (CKD) patients, and PWIDs must be checked with HCV ELISA regularly and remain under strict follow-up.
- People with HIV must strictly remain under the observation of a clinician. Because HCV-HIV coinfection progresses more rapidly. Before HCV treatment, HIV infection

must be suppressed because drug-drug interaction (DDI) may take place with DAA treatment of HCV.

- All patients who have achieved SVR must also be followed for liver check-ups periodically to ensure their health condition. Patients who are unable to achieve SVR must be periodically checked for HCC progression. Re-infection often occurs post-SVR achievement thereby patients should again be checked for RNA testing. If re-infection occurs re-treatment should be recommended.
- Patients with dialysis must also be managed cautiously because side effects of DAA may be a complex problem for them with renal injury.
- Patients co-infected with HCV and HBV or HCV and tuberculosis (TB) must be cautiously treated because possible drug-drug interaction may take place. It is recommended if a patient is co-infected with tuberculosis and HCV, then tuberculosis must be cured first then HCV treatment should happen.
- DAAs treatment with pregnancy is not recommended, therefore pregnant women must be screened for HCV. If an active HCV infection is found then contraception may be recommended after therapy.

Chapter 2

2 Finding problems and Knowledge Gaps:

Population-based HCV prevalence is not well reported in India, especially in West Bengal (23). Besides HCV genotype and subtype distribution in West Bengal is not very well reported (58). Moreover, there is no well-documented literature exists about the mutation rate and the geographic and evolutionary relationship of the Indian HCV strain with neighbouring countries. Very recently new HCV genotype (Genotype-8) has been isolated from Punjab, India (8). Previous reports showed that the north-east states of India also had unique subtypes 6n and 6k (31,59). It may be possible that West Bengal has such unique HCV subtypes. HCV genotype is also very important for DAAs treatment (60). Therefore, epidemiological and genotype distribution data will help manage HCV infection and future drug development. Yet, there are very limited studies which focus on these problems.

Other than this not all HCV infections become chronic, 20-30% of patients can clear the virus infection spontaneously, and some patients can face liver cirrhosis which ultimately leads to HCC. HCV Genotype 3 is associated with more steatosis and fibrosis (61).

Besides, HCV has a high mutation rate, ranging from 3.5×10^{-5} to 1.2×10^{-4} base substitutions/site every year (62). Resistance-associated mutations (RAS) are also reported from the DAAs era (63). RASs in the HCV genome are hypothesised to be created as a consequence of spontaneous mutations in the HCV RNA (genome), and some HCV isolates with RASs can evolve in response to the selection pressure of DAAs (64). The high mutation rate of HCV also leads to HCV quasispecies which also helps in viral persistence and chronic infection (65). Therefore, mutational studies are critical and can reveal some underlying

mechanisms of disease progression. There were also plenty of reports that talked about the relationship between HCV NS3 protein with cancer progression (66–68). However, how NS3 gene mutation can affect disease progression is not clear. Besides, there are also knowledge gaps about naturally occurring DAA-RAS.

Moreover, there is no vaccine for HCV till now because they have vast genetic variability. Viral NS3 also showed a good immune response with potential viral clearance (69–71). It is not clear if there is any conserved region in HCV proteome which can be targeted for therapeutic intervention and suitable for future therapeutic targets. Previous studies showed that the NS3 protein region of HCV has long been targeted for HCV therapy. This protein of HCV is a bifunctional enzyme, it acts as protease and helicase. Previous reports also showed helicase region is conserved and can also be a potential target (72,73). Immunisation with NS3 protein also gave rise to promising immune responses (74). Recently developed epitope prediction and reverse vaccine modelling have become very popular. Yet there is a paucity of information about implementing this method in HCV epitope prediction and validation.

Chapter 3

3 Review of literature:

3.1 Background:

3.1.1 Historical Perspective and Taxonomy of HCV virus:

Hepatitis A and B were discovered long ago before the hepatitis C virus. Scientists found that a patient had mild symptoms of hepatitis at first but later it developed hepatitis-associated symptoms with a longer incubation period. Eventually, physicians found more patients with similar symptoms and they were quite sure that this infection was not because of Hepatitis A or B. So, they termed it as "Non-A, Non-B hepatitis." But they could not isolate or describe it. In 1989, Charles Rice and his co-workers were able to isolate an RNA virus from this type of patients and describe it and termed it as the "Hepatitis C virus". For their discovery, they also received the "Noble Prize" in the year 2020 (75). HCV is a single, positive-stranded RNA virus. The Hepatitis C virus belongs to the Flaviviridae family and comes under the Hepacivirus genus. High genetic variability of HCV leads to 8 genotypes and more than 86 subtypes (9).

3.1.2 HCV virion, genome organization and function:

HCV is a positive-stranded RNA virus. It has a 9.6 kb long single-stranded RNA genome. This 9.6 kb RNA has both ends (5′ and 3′) flanked with UTR (Untranslated Region). HCV RNA codes for a polyprotein which is approximately 3,010 amino acids long. This polyprotein will finally give rise to 10 proteins among which 3 are structural proteins (Core, Envelope1, and

Envelop2), 1 is a channel protein (p7) and 6 are non-structural proteins (NS3, NS4a, NS4b, NS5a, and NS5b) (76). A brief detail of those regions and proteins is discussed below-

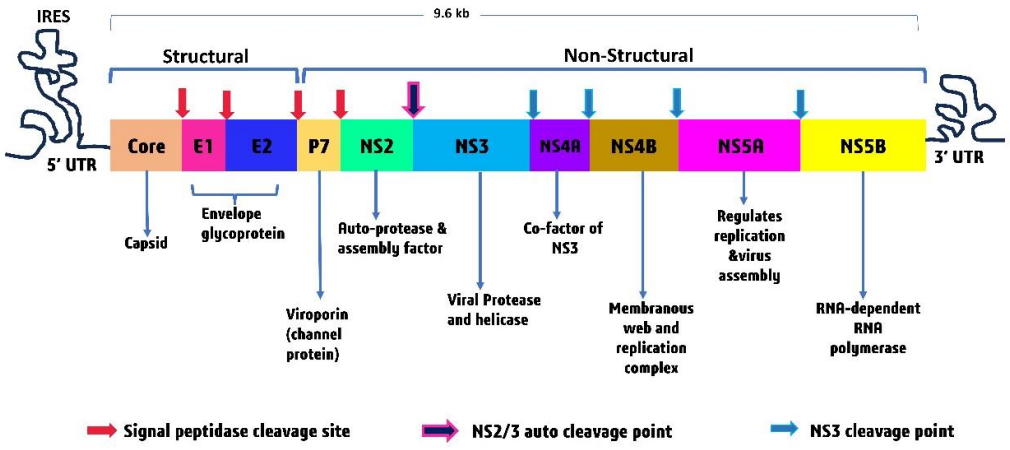

Figure 4: Schematic diagram of genetic organization of HCV genome.

3.1.2.1 5′ UTR:

The 5′ UTR region is flanked upstream of the HCV open reading frame (ORF). It consists of about 341 nucleotides which form four highly structured domains consisting of stem-loops and pseudoknots (I, II, III and IV) (77). First 1-43 nucleotides make a proximal stem-loop (78). Domain II, III and IV with 12-30 nucleotides of domain I make the Internal ribosome entry site (IRES) (79). This IRES helps HCV to directly load host ribosomes into RNA and initiates polyprotein translation. 5′ UTR region is a very conserved region and acts as a cis-acting region. Mutations in this region can hamper the HCV cell cycle (78,79). Electron microscopic study reveals that domains II, III and IV make distinct structures, and there is also a hinge present over domains II and III (80). Atomic force microscopy also proves that IRES has an Mg2+-based switch which can induce “open” or “close” conformation for ribosomes (81).

3.1.2.2 Core:

The HCV core protein is 191 amino acids long structural protein. It is basic in nature (82). It is a multifunctional protein and has immense importance in host pathogenesis. It’s mainly

responsible for forming capsid of the HCV virus and protecting the genomic RNA of HCV. It is a monomeric protein. It has the capacity for lipid binding and RNA binding (83). The molecular weight of mature core protein is 21kDa. The core protein contains 3 different domains (D1, D2, and D3). Domain 1 (D1) is highly cationic, consists of 117 amino acids, and is the major domain which forms capsid. Domain 2 (D2) of core protein is a hydrophobic domain which is very well known for its role in lipid binding(84). Domain 3 (D3) is also hydrophobic in nature. D3 interacts with endoplasmic reticulum (ER) and helps in anchoring (85). The core protein is also responsible for increased reactive oxygen species (ROS) generation and inhibits the electron transport chain in mitochondria (86). Core protein also interferes with nuclear localization signal (NLS) (87). The core protein is also found to be circulated in the bloodstream (88). The development of HCC may be attributed to the core protein-induced dysfunction of key pathways' components, such as p53, AP-1, MAPK extracellular signal-regulated kinase-extracellular signal-regulated kinase, transforming growth factor β, vascular endothelial growth factor, Wnt/β-catenin, cyclooxygenase 2 (COX-2), and peroxisome proliferator-activated receptor α (PPARα) (89–92).

3.1.2.3 HCV Envelope (E1 and E2):

HCV envelope protein is composed of two subunits, Envelope 1 (E1) and Envelope 2 (E2).

Envelope 1 (E1) is a glycosylated subunit of HCV envelope protein. This is a transmembrane protein which plays a role in virus entry. It is composed of 192 amino acids. Interestingly, bioinformatic analyses revealed that it has a conserved region. It has 4 different domains- a. N-terminal domain (NTD, 192-239), b. putative fusion peptide (pFP, 272-285), c. conserved region (CR, 302-329) and d. a c-terminal transmembrane domain (TMD, 350-381). Studies revealed that NTD may be exposed on the protein surface. NTD may have crosstalk with TMD and facilitate the complex formation with E2 (93). The pFP domain has a role in viral envelope

fusion with the host membrane during HCV entry (94,95). CR region is hydrophobic but its role is not clear.

Envelope 2 is also a type I transmembrane protein like E1. This is also a structural glycoprotein but it has a much greater size than E1. HCV E2 is composed of approximately 365 amino acids and the relevance of E2 for viral entry when linked to E1 is tremendous. Scientists have long been interested in HCV E2 because of its unsolved structure. Later it was solved by two independent groups (96,97). The structure determination was challenging because of the presence of hypervariable regions (HVR). Therefore, the crystal structure of E2 was further improved by Electron microscopy to understand the full structure of E2. Interestingly, the E2 structure resembles the Immunoglobulin protein (Ig) like β-sandwich structure. From the crystal structure, it was revealed that it has one hydrophobic back layer and one basic front layer (94). E2 contains several hypervariable regions which are HVR1 (aa384-411), HVR2 (aa461-481), HVR3 (aa431-466) and IgVR (aa570-580) (98–101). The versatility of this region may evolve due to the host immune system (102). The HVR1 region of E2 also interferes with the low-density lipoprotein receptor (LDL-R) and enables virus entry through the scavenger receptor, class B type I(SR-BI) (103).

3.1.2.4 P7 (channel protein/Viroporin):

The HCV p7 protein is a tiny, integral membrane protein of 63 amino acids that is encoded at the junction of the HCV polyprotein's structural and non-structural regions. Although most HCV polyprotein precursor cleavages occur during or immediately after translation, cleavages at the E2-p7 and p7-NS2 sites are delayed, resulting in the formation of an E2-p7-NS2 precursor (104). When expressed alone in mammalian cells, epitope-tagged p7 has been shown to enter the ER membrane with two membrane-spanning helical domains, both N- and C-termini faced towards the ER lumen, and a short hydrophilic loop facing the cytoplasm (105). However, alternate theory may also be possible where C-termini were found to face towards

the cytoplasm (106). Electron microscopy studies revealed that p7 may consist of multi-monomeric form which eventually forms hexamer or heptamer(105,107). Recent high-resolution Electron microscopy studies found that the structure of p7 is like a flower and six protruding petals oriented towards the ER lumen (108). A recent NMR study revealed an unexpected N-terminal α-helix is connected to the first transmembrane helix (TM1) via a short turn. Moreover, a long cytosolic loop extending from residue 33 to 39, including the di-basic motif connected TM1 and TM2 (109). *In-vitro* studies showed that p7 forms oligomers and shows that it can efficiently transport ions across the artificial membrane (109–111). HCV p7 is also an essential protein for virus assembly and production of progeny virus (112). New studies found it can also be a good target for therapeutic intervention (113–115).

3.1.2.5 NS2 protein:

NS2 is a non-structural protein. It consists of 217 amino acids and the molecular weight of this protein is about 23kDa. It is also a transmembrane protein having a carboxy-terminal cytoplasmic domain and a hydrophobic amino-terminal subdomain comprising up to three putative transmembrane segments. Expression of NS2 protein in cells is tightly regulated and protein turnover may vary across the HCV genotypes (116,117). The NS2-3 protease is formed from the C-terminal domain (residues 94 to 217) of NS2 and residues 1-181 of NS3 (118). Studies revealed that NS2 is extremely important for the HCV life cycle (119). Solid-state NMR was used to determine the structure of NS2's first transmembrane segment, which revealed a flexible helix in the N-terminus (residues 3-11) coupled to a stable alpha helix (residues 12-21) by two glycine residues (119). NS2 was also found to interfere with various host signalling like apoptosis (120), cell cycle arrest (121), cAMP signalling (122), cytokine signalling (123), fatty acid metabolism in the liver (124), RNA interference (125), adapter proteins in Golgi-complex (126) and so on. NS2 can also thought to be a potential antiviral target for future endeavours (127).

3.1.2.6 NS3 protein:

The most important and interesting protein of the HCV proteome is the NS3 protein. Its' molecular weight is almost 70kDa. Therefore, it's also known as p-70. It consists of 631 amino acids. The interesting feature of this protein is that this protein is a bifunctional enzyme and can act as both a protease and a helicase. The first 180 (N-terminal) amino acids make viral protease rest of the amino acids form the helicase.

N-terminal protease: NS3 protease comes under the trypsin/chymotrypsin superfamily, to be specific it is a serine protease (128,129). The enzyme is made up of two β-barrel domains sandwiched between two short α-helices—one of the β-strands of the N-terminal β-barrel produced from the central hydrophobic region of NS4A. A Zn2+ ion stabilises the structure, which is coordinated by three cysteine residues and one water molecule (130). Protease activity necessitates the presence of a catalytic triad (Ser-139, His-57, and Asp-81) as well as an

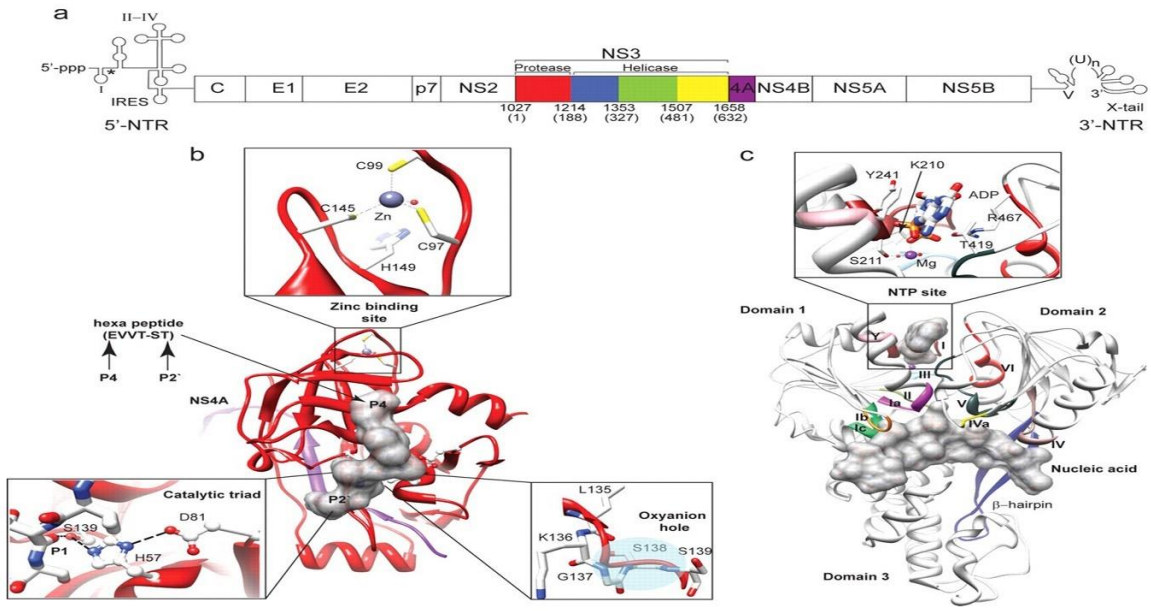

Image courtesy: Raney KD, Sharma SD, Moustafa IM, Cameron CE. Hepatitis C virus non-structural protein 3 (HCV NS3): a multifunctional antiviral target. J Biol Chem. 2010 Jul 23;285(30):22725-31. doi: 10.1074/jbc.R110.125294. Epub 2010 May 10. PMID: 20457607; PMCID: PMC2906261

Figure 5: Position and structure of HCV NS3 protein. (a) Relative position of HCV NS3 protein. (b) NS3 catalytic triad, Zinc binding site and oxyanion hole. (c) NS3 NTP site.

oxyanion hole (backbone amides of Gly-137 and Ser-139) (131). The catalytic/chemical mechanism of NS3 protease-catalyzed peptide bond cleavage is most likely the same as that seen for other serine proteases (132).

C-terminal helicase: Based on sequence homology, helicases are divided into superfamilies (SFs), with SF1 and SF2 being the most numerous (133,134). With an equilibrium dissociation constant in the low nm range, a binding site size of 7-8nt, and little or no known cooperativity, NS3 binds to DNA and RNA (135). At pH 6.5, binding to RNA and DNA, as well as unwinding of both substrates, is increased (136). It is unclear if the pH requirement reported in vitro has biological importance, but it might point to a distinct environment within the membranous web where HCV replication occurs (137). In vitro, NS3 interacts with itself to create huge aggregated structures, however, it is unknown whether this oligomerization is physiologically relevant (138). The active form of NS3 may be a monomer (139), dimer (140) or oligomer (138). Evidence suggests that monomeric NS3 may unwind RNA quickly but with poor processivity (139,141). When numerous molecules attach to the same DNA substrate molecule, NS3h unwinding activity increases. This is known as functional cooperativity, and it occurs when all the attached enzymes move in the same direction on the substrate's tracking strand (142).

3.1.2.7 NS4a protein:

HCV NS4a protein is a non-structural, single-pass transmembrane protein that functions as a co-factor of NS3 protein. It is the smallest of all non-structural proteins (143). It consists of 54 amino acids and the molecular mass of this protein is 16kDa (144). The sequence of NS4a protein is very conserved which indicates that it may take part in protein-protein interactions. The NS4A protein has many important functions in the HCV life cycle, including (a) attaching the NS3-4A complex to the outer leaflet of the endoplasmic reticulum and mitochondrial outer membrane, (b) serving as a cofactor for the NS3A serine protease, (c) augmenting NS3A

helicase activity, and (d) regulating NS5A hyperphosphorylation and viral replication. The interactions between NS4A and NS4B control genome replication and between NS3 and NS4A play a role in virus assembly (145).

3.1.2.8 NS4b protein:

NS4B is a 261aa integral membrane protein with ER or ER-derived membrane localization. The molecular mass of HCV-NS4b protein is about 27kDa. It is a hydrophobic protein. Studies postulated that NS4b has an N-terminal amphipathic helix (146). This amphipathic helix (AH) was conserved across all HCV isolates, indicating that it plays an important role in productive spontaneous infections. The introduction of mutations targeted to damage the hydrophobic face of the AH rendered it unable to mediate membrane attachment. A putative nucleotide binding motif (NBM) was discovered in the NS4B main sequence, starting in the centre of the protein (147). NS4b was also found to be bound with GTP like other nucleotide-binding proteins (148). When expressed independently in mammalian cells NS4b protein was found to be localised on ER membranes (149). NS4b also plays a crucial role in viral replication (150). It is also thought to be an important factor in inducing carcinoma in the host by interfering with the PI3-Akt pathway (151). It is also an important protein which helps HCV bypass the host immune system (152). It also interferes with lipid metabolism(153). It has also contributed to HCV-induced 'Membranous Web' (154). Studies also showed that NS4b protein can be a potential target for therapeutic intervention of HCV (155,156).

3.1.2.9 NS5a protein:

NS5a protein is a non-structural protein, that is very essential for the HCV replication complex (157). The molecular mass of HCV-NS5a protein is 49kDa. It consists of 447 amino acids. It is composed of three domains: I, II and III. The domains are separated by low-complexity sequences (linkers), which have flexible structures (158). Studies proved that it may take part in the phosphorylation reaction at the time of replication (159). It also has a zinc-binding motif

which falls in domain I of the protein (158). Domain I of NS5a also has an amphipathic helix that helps NS5a to interact with the ER membrane (160). Domains 2 and 3 appear to constitute an inherently unstructured portion of the protein and have functions in RNA replication and the assembly of hepatitis C virus particles (161,162). The crystal structures of genotype 1b NS5A domain 1 (NS5A-D1) show that dimers form during crystallisation. However, the creation of multimers or protein-protein interactions is a necessary result of protein crystallisation (163). Other than replication, NS5a is also very important for virion morphogenesis and assembly (164). It has also been found to interact with other HCV proteins like core (165), NS5b (166), NS4b (167), NS3 protein (168). NS5a was also found to have interacted with the host factors for infection establishment and HCC development (169–171). NS5a is also an immensely important protein because it's often targeted by HCV-mediated DAA therapies (172–174).

3.1.2.10 NS5b protein:

NS5b protein of HCV is a non-structural protein which serves as RNA-dependent RNA polymerase (RDRP). It is composed of 591 amino acids and the molecular mass of this protein is 66kDa. Till date near about 100 crystal structures of HCV-NS5b have been reported from various genotypes (1a, 1b, 2a and 2b) but all of them lack a c-terminal membrane-anchoring tail (175). The structure of NS5b is almost like a right hand which consists of palm, finger, and thumb domains (176,177) and appears like a closed fist. Extensive efforts to build a high-resolution crystal structure of wild-type HCV polymerase in association with developing RNA primer-template pairs have been fruitless. However, a structure has been described with a polyuridine template in an unproductive conformation (178). According to crystal models of NS5B, Glu-18 is situated amid a lengthy loop linking the fingers and thumb, which is unusual among RdRPs. The template RNA appears to be located near the linking section of this loop. His-502 is located in helix T, which pairs with helix U in the thumb subdomain. The paired helices are one of the armadillo repeats, which are unique among RdRPs and contribute to the

thick thumb of HCV NS5B (179). Both Glu-18 and His 502 are critical for RDRP activity (180). Studies revealed that HCV-NS5b may interact with other HCV proteins (181–183). The error-prone nature of HCV NS5b is one of the factors which influences high genetic diversity (184). It is also one of the most significant antiviral targets for DAA therapies and is extensively studied for inhibition (185,186).

3.1.2.11 3′ UTR:

UTR is flanked downstream of HCV-ORF. It contains about 225 nucleotides. It is also conserved and highly stem looped like 5′ UTR. It also contains a 98 bp long 'X' region which plays an important role in the IRES-dependent translation of HCV RNA (187). This 'X' region plays a significant role in switching between translation and transcription of viral RNA(188). Researchers revealed that 3′ UTR can take two types of conformation – a. two stem model (comprises SL1' and SL2' structure) b. three stem model (comprises SL1, SL2 and SL3 structure). Where SL stands for stem-loop structure (189). This 3′ UTR also contains 'poly-U' sequences which might play a role in HCV replication (190). Studies also revealed that 3′ UTR might play a role in viral encapsidation (191). HCV 3′ UTR preserves ribosome complexes upon translation termination, facilitating the effective start of successive rounds of translation (192).

3.2 High-risk group populations for HCV infection:

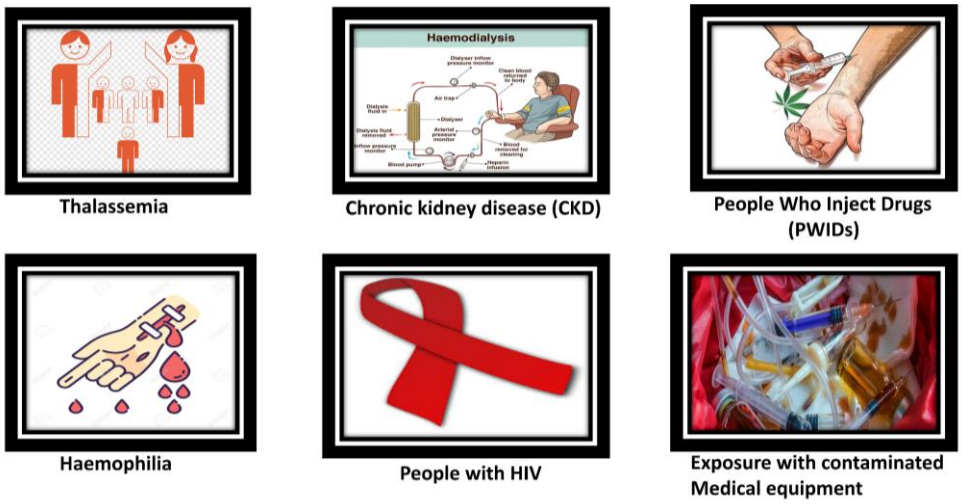

Figure 6: Various risk group populations for HCV infection.

3.2.1 Thalassemia:

Thalassemia is a hereditary haematological condition caused by defective haemoglobin production. The term is taken from the Greek word "thalassa" meaning "the sea", as the illness was initially recorded in populations living near the Mediterranean Sea. Another term encountered in literature, albeit uncommon, is Cooley's anaemia, after the name of Prof. Cooley Thomas, a paediatrician in the United States who first described the clinical symptoms of this condition. This is a congenital autosomal recessive haemoglobin (Hb) disorder that has a significant occurrence in the Indian subcontinent, Mediterranean and Middle Eastern nations, and Southeast Asia(193).

Thalassemia is a spectrum of illnesses that can vary from barely noticeable blood abnormalities to severe or deadly anaemia. Adult haemoglobin is made up of two alpha (α) and two beta (β) polypeptide chains. The haemoglobin alpha gene (HBA1 and HBA2) encodes the α-chain and is found on chromosome 16. The β-chain is encoded by the haemoglobin beta gene (HBB), which is on chromosome 11(194,195).

Alpha thalassemia is caused by the loss of the alpha-globin gene, which leads to decreased or non-existent synthesis of alpha-globin chains. The alpha globin gene has four alleles, and the severity of the condition varies depending on how many alleles are deleted. The most severe type is four allele deletion, which produces no alpha globins and causes excess gamma chains (existing throughout the embryonic stage) to form tetramers. It is not compatible with life and causes hydrops fetalis. One allele deletion is the mildest variant and is usually clinically silent (194).

Beta thalassemia is the outcome of point mutations in the beta-globin gene. It is classified into three groups according to the zygosity of the beta-gene mutation. A heterozygous mutation (beta-plus thalassemia) causes beta-thalassemia minor, in which beta chains are underproduced. It is often mild and asymptomatic. Beta thalassemia major is caused by a homozygous mutation (beta-zero thalassemia) in the beta-globin gene that results in the complete lack of beta chains. It appears clinically as jaundice, growth retardation, hepatosplenomegaly, endocrine problems, and severe anaemia necessitating lifelong blood transfusions. The condition in between these two categories is called beta-thalassemia intermedia with mild to moderate clinical signs (194).

3.2.1.1 Challenges of HCV infection in thalassemia:

Thalassemia patients are at risk for transfusion-transmitted viral infections, including hepatitis C virus, due to their requirement for blood transfusions throughout their lives (HCV). Infection with HCV results in chronic infection in a large proportion of individuals, and the prevalence rate of chronic hepatitis C in patients with thalassemia major in the EMRO (WHO Regional Office for the Eastern Mediterranean) region ranges from 11% to 69% depending on the patient's age and the local seroprevalence of HCV(196). Although the natural history of chronic HCV infection in thalassemia patients is unknown, the patient's morbidity and mortality are expected to be higher. Liver disease is more severe in thalassemia patients and can be

exacerbated by hepatic siderosis. In transfusion-dependent thalassemia, hepatic iron overload can worsen liver disease by causing inflammation and fibrosis. It also reduces responsiveness to interferon-based treatments (197–199).

3.2.1.2 Prevalence of HCV in thalassemia population – a global and Indian scenario:

As thalassemia is not prevalent globally. It is majorly an Asian continent problem; a problem affects mostly third-world countries. However, thalassemia also spread to 1st world countries due to immigration and other humanitarian factors (200). The chances of HCV infection in thalassemia are very high. Therefore, this population is one of the contributors to the global HCV burden. In various studies from diverse populations of thalassaemic patients (United States, Italy, and Greece), the frequency of cirrhosis ranged from 10 to 20%, and the incidence of HCC in thalassemia patients was gradually increasing (199). The epidemiology and genotype distribution of HCV infection in thalassaemic people are not extensively documented in India or other countries, particularly HCV genotypes and geographic distribution. Determination of HCV genotypes and subtypes is crucial to determine the source of HCV infection in a particular population and it is also required for clinical management, therapeutic intervention, and development of an effective HCV vaccine. A hospital-based investigation on the prevalence of anti-HCV in thalassemia patients was undertaken (201), however, there was a lack of a complete study on the HCV viremia and genomic diversity of HCV in the β-thalassaemic population from India.

3.2.2 Chronic kidney disease (CKD):

Chronic kidney disease (CKD) is a disorder that causes a progressive decrease in kidney function over time. It involves renal injury and a decreased capacity to filter. As a result, poisonous compounds, waste products (creatinine and urea), excess water, and excretory

wastes collect in the body, potentially leading to a variety of issues such as high blood pressure, anaemia (low blood count), weak bones, poor nutritional health, or nerve damage. Early identification and treatment can frequently prevent chronic kidney disease from progressing. When kidney disease worsens, it may eventually lead to renal failure, which needs dialysis or a kidney transplant to stay alive (202).

There are five stages of kidney damage, ranging from very mild damage in Stage 1 to full kidney failure in Stage 5, which can be used to determine the exact extent of kidney damage. CKD often generates no symptoms in its early stages. Glomerular filtration rate (GFR) is a measure of kidney function. The typical GFR is 100 to 140ml/min in men and 85 to 115ml/min in women. As renal disease worsens, GFR decreases. CKD often generates no symptoms in its early stages (203).

Stages	Description	GFR ($ml/min/1.73m^2$)
Stage 1	Slight kidney damage with normal or increased filtration	More than 90
Stage 2	Mild decrease in kidney function	60 to 89
Stage 3	Moderate decrease in kidney function	30 to 59
Stage 4	Severe decrease in kidney function	15 to 29
Stage 5	Kidney failure	Less than 15 (or dialysis)

Table 1: Different Stages of Chronic Kidney Disease according to the GFR. (Adapted from https://www.kidneyfund.org/kidney-disease/chronic-kidney-disease-ckd/)

Stage 5 chronic kidney disease is also known as kidney failure or end-stage kidney disease or end-stage renal disease (ESRD), wherein there is total or near total loss of kidney function.

Most individuals onward stage III of kidney disease need dialysis every week or kidney transplantation (KT) to stay alive (204).

3.2.2.1 Challenges of HCV infection in CKD patients:

CKD patients are at high risk for HCV infection due to repeated haemodialysis of CKD patients. HCV also complicates CKD management by augmenting ESRD. It also plays an important role in morbidity and mortality in CKD patients, and the risk of HCV-mediated death in stage-V haemodialysis patients is particularly very high (205). HCV-related kidney disorders can appear clinically as nephritic, nephrotic syndrome, or isolated proteinuria, with or without renal function impairment. Pathological abnormalities on renal biopsy include membranoproliferative glomerulonephritis (MPGN), membranous glomerulopathy (MG), IgA nephropathy, focal segmental glomerulosclerosis, mesangial proliferative glomerular nephritis, and tubulointerstitial nephritis (206). Another study shows HCV is also associated with cryoglobulinaemic glomerulonephritis in CKD patients (207). Therefore, HCV infection in the CKD population needs to be managed carefully.

3.2.2.2 Prevalence of HCV infection in CKD patients- a Global and Indian Scenario:

HCV is one of the major risk factors for CKD patients. In 2004, the Dialysis Outcomes and Practice Patterns Study (DOPPS) found that 13.5% of hemodialysis patients have hepatitis C. Hepatitis C prevalence rates among these individuals change with geographical area, with less than 5% in the United Kingdom and Germany and more than 20% in Spain and Italy (208). In another meta-analysis report, four hundred and seven studies with 1,302,167 individuals were evaluated for HCV prevalence around the world. The overall pooled prevalence was found to be 21%. The worst scenario was observed in Africa (about 28%) (209). Very few studies discuss about HCV prevalence among CKD patients in India (210,211). Clearly, this is not adequate to

understand the overall scenario of India. Therefore, an urgent need for studies to estimate the HCV disease burden in the CKD population.

3.2.3 People who inject drugs (PWIDs):

People who inject drugs (PWIDs) represent a key issue in controlling the spread of HCV across the world, causing a socioeconomic and health burden by squandering precious human resources. According to the World Health Organization, globally, around 130 million individuals who inject drugs (212). In third-world nations like India, where the number of IDUs was supposed to be quite low. which is a mistake (213,214). There are about 0.2 - 1.1 million people in India who inject drugs (215). Hepatitis C virus poses a significant concern to PWIDs because it spreads through blood-to-blood contact and the use of an infected needle.

3.2.3.1 Challenges of HCV infection in PWIDs:

Numerous hurdles exist at the patient, provider, and healthcare system levels that have prevented PWIDs from obtaining prior HCV treatments, including interferon-based medications, which were less successful and had major patient adverse effects. Patient-level barriers include a low understanding of HCV and the potential for morbidity and death in the absence of symptoms, worries about the side effects of therapy, and general difficulties with accessing the healthcare system (216,217). Provider-level hurdles include a lack of understanding about HCV and its therapies (since HCV therapy was typically only available in specialist care settings) and a reluctance to treat PWIDs owing to fears about reinfection (218,219). System-level hurdles, such as a restricted number of centres for HCV testing and treatment, as well as limits on access to HCV treatment based on drug or alcohol abstinence criteria and liver disease stage, have also been identified (220).

3.2.3.2 Prevalence of HCV among PWIDs-a global and Indian scenario:

Globally, more than 50% of PWIDs are infected with HCV (221). The risk of HCV infection is greater among young PWIDs than the older ones. Women-PWIDs are also at high risk of HCV infection (222). Trending of HCV infection among PWIDs is found to be increasing in the USA (223). In EU/EEA countries HCV viremia was also found to be very high (224). HCV prevalence in Central Asian countries among the PWIDs population was also found to be high (225).

Although India is sandwiched between the 'Golden Crescent' and 'Golden Triangle', prevalence data of HCV among IDUs are not very well established; however, it can be concluded from various studies that HCV seroprevalence among IDUs is moderate (30-50 %) throughout India, but there are also some pockets with high HCV prevalence among IDUs (60-90%) (226,227). However, there are some studies which show distinct patterns of geographical distribution, unusual genotypes, and recombinant strains in the Indian PWIDs population (31,33,59,226).

West Bengal, which serves as a corridor for eastern and northeastern India, has been considered to have a low prevalence of HCV infection for a long time (228). There is only one documented HCV sero-survey report among IDUs in the northern part of West Bengal particularly in Darjeeling and its adjacent districts (229). There are over 5000 IDUs in Darjeeling and injecting techniques are very common (214,230). Therefore, the prevalence of HCV and genotype distribution among IDU patients in West Bengal are very important for future endeavours.

3.2.4 Other Risk Group Populations for HCV Infections:

Other risk groups include haemophilia and people with HIV. Haemophilic children who often receive blood factors (VIII and IX) have a high chance of HCV infection. Although very limited reports are available on this (231–233). In India HCV prevalence in the haemophilia population is still underreported.

People with HIV are also at high risk for HCV infection due to shared routes of transmission. About 25-30% of cases of HIV-infected patients co-infected with HCV (234). HIV co-infects with HCV often become very hard to manage because both drugs may interfere during the treatment (235).

Although these population groups are very hard to find HCV prevalence in these groups is an important factor for future HCV control programs.

3.3 HCV infection among general population groups:

The general population became infected with HCV because of nosocomial contamination of blood and blood products, contaminated medical and surgical equipment, or tattooing and homosexual relations. Most of the time individual faces mild symptoms at the beginning and remains undiagnosed (236). But within 10-15 years that person develops liver cirrhosis or chronic liver disease (CLD) (5), some of which eventually lead to decompensated liver cirrhosis and HCC. Nonetheless, region-specific HCV epidemiological investigations were lacking in the case of chronic liver disease in India (23). Although a few studies were there HCV genotype distribution and viremia remain still shady in the present situation (237–240).

3.4 Rapid mutation rate of HCV - A ‘Swiss army knife’ for HCV survival:

A prominent characteristic of HCV infection is its’ genetic diversity. HCV does have an error-prone RNA-dependent RNA polymerase (RdRp) which yields a high rate of mutations in HCV (241,242). These mutations are the main contributing factor behind HCV survival in the host system and against other challenges.

3.4.1 How HCV mutations help in immune invasion:

The sequence variability of HCV not only varies from person to person but also variability exists and accumulates within the same individual with time. Therefore, in each infected host, there are a swarm of genetically resembled but distinct virus variants, which are often termed quasi-species of HCV (243,244). This distinctively diversified combination of viruses in an individual is not fully random but rather appears to be directed by the host immune system and balanced by functional restrictions (245). These high rates of mutations confer selective advantages to the virus by which the virus invades the immune system and increases replicative fitness (246,247). Because immune responses develop over weeks and pathogens proliferate over the range of hours or days, it is understandable that immune escape mutations may reduce the efficiency of the immune response (248,249). The fast rate of virion turnover, along with the lack of proofreading by the HCV RNA polymerase, causes numerous changes in the viral genome. Mutations in class I or II MHC-restricted T cell epitopes may change the outcome of infection by blocking or delaying the clearance of infected hepatocytes (250). Longitudinal investigation during chronic infection revealed a very minimal rate of amino acid alteration in CTL epitopes, indicating that CTL escape may be confined to early infection (251).

3.4.2 How HCV mutations affect the therapeutic management:

HCV mutations also interfere with the outcome of HCV-related therapies. Studies have found that viral mutations play a major role in SVR achievement in the case of interferon therapy (252). It was also proved that HCV mutates itself to cope with Ribavirin treatment (253). HCV-resistant associated substitution (RAS) mutations were also found against protease inhibitors (254–257). NS5b inhibitor-resistant associated substitutions also came into light (258,259). NS5a inhibitor-resistant mutants were also reported (260,261). Naturally occurring resistant-

associated mutations also emerged (262). More about DAA-RAS lists and articles can be found here- http://hcv-glue.cvr.gla.ac.uk/#/project/rap.

Therefore, mutational analysis of HCV is crucial for understanding disease biology, drug development and vaccine development. By mutational analysis, we can also predict the outcome of DAA therapy, we can also find out where the conserved regions lie which can be a potential target for future therapeutic management of HCV.

3.5 Importance of HCV NS3:

3.5.1 NS3 protein and hepatic oncogenesis:

The carcinogenic effects of the HCV NS3 protein are less reported than those of the HCV core protein. The NS3 protein is considered to interfere with normal cellular activities, such as cell proliferation and cell death, and is involved in HCV carcinogenesis. Research has identified the NS3 secondary structure as a potential risk factor for HCC development (263). The NS3 protein has been shown to interact with proteins involved in cell cycle and/or apoptosis regulation. Some subgroups of HCV-1b isolates have been associated with HCC development. The involvement of the NS3 protein has been highlighted in the progression of hepatic cirrhosis (264), which is itself a risk factor for HCC development. Several studies also investigate the link between NS3 protease mutations and HCC development (265,266). NS3 protein interferes with p53 signalling (66), and the NS3 protein increases hepatocellular carcinoma cell migration by boosting PPM1A ubiquitination and degradation (68). It was also found to interfere with apoptosis signalling by upregulating MAP kinase, c-Jun NH2-terminal kinase (JNK) signalling pathway (267,268). NS3 protein promotes cancer cell invasion by activating matrix metalloproteinase-9 and cyclooxygenase-2 through ERK/p38/NF-κB signal cascade (269).

3.5.2 NS3 protein helps HCV in immune invasion:

Pathogen recognition receptors identify pathogen-associated molecular patterns (PAMPs), which include nucleic acids, proteins, carbohydrates, and lipids (PRRs). Toll-like receptors (TLRs) are PRRs found on the cell surface that recognise extracellular PAMPs; however, certain TLRs also detect intracellular PAMPs. HCV-NS3protease cleaves TLR3 receptors (TRIF) and thereby escapes from the host immune system (270). HCV NS3/4A cleaves mitochondrial antiviral signalling (MAV) off the mitochondria to evade innate immunity(271). HCV NS3 protein suppresses TNF-α-induced NF-κB activation via interacting with the linear ubiquitin chain assembly complex (LUBAC) (272). Therefore, the NS3 protein plays a major role in bypassing the immune system during HCV infection establishment.

3.5.3 NS3 protein helps in virus assembly:

Studies have found that NS3 is essential for the generation of fast-sedimenting core-containing particles, which are considered to represent non-infectious virions that will undergo further phases of maturation (273,274). Another study showed HCV NS3 protein helicase part may play a role in virus assembly (275).

3.5.4 NS3 Protein and HCV replication:

NS3 protein is essential for viral replication. It is proved that HCV NS3 (helicase part) can unfold viral G-quadruplex RNA structures (276). It is also found that the hydrophobic content of NS3 helix α-(0) is crucial for HCV replication and infection(70). The linker region between NS3 protease and helicase is also important for Viral replication(277). It is also proved that NS3-4a associates with NS5a and NS5b to form replication organelles (RO) for HCV(278).

3.5.5 NS3 as an anti-viral target:

Viral protease has long been targeted for HCV treatment (257,278,279). Although NS3-related antiviral resistance also came into play (280). Recent studies also proved that NS3 helicase can also be a target for antiviral therapy (72,73,281). The HCV NS3 protein was found to be a prominent target for vaccine development against HCV (69,282). The NS3-specific T-cell response is also linked to viral clearance (74,283). It has also been found that the NS3 protein of HCV can trigger T-cell responses better than other proteins (71,284).

Thus, HCV-NS3 protein can be a good target for therapeutic and vaccine development.

3.6 Ups and downs of HCV vaccine development:

The introduction of all oral, interferon-free direct-acting antivirals (DAAs) has reshaped the therapeutic management of HCV, especially in developed countries. Although DAAs have sparked excitement for global HCV control, some treated patients have developed resistance to DAAs, and the spread of resistant HCV genotypes was recorded in clinical studies even before DAAs were licenced (285). In recent days the situation has become more sensitive (286). A successful preventative vaccination will reduce HCV incidence significantly, paving the way for global HCV control.

3.6.1 Suitability of traditional approaches for HCV vaccine design:

Live-attenuated and inactivated whole virus vaccines have proven successful against other viruses, but neither technique is practical for developing HCV vaccines. The inability to culture HCV until recently, as well as the current limitations of HCV culture techniques, have hampered the development of a live-attenuated or inactivated complete HCV vaccine. Live-attenuated vaccines against other viruses have been developed in two ways: by passing the virus via nonhuman primate cell lines, which can produce natural variations that limit

replication in human cells, and by genetic deletion or inactivation of virulence components. However, HCV does not replicate significantly in nonhuman primates cell lines, and HCV virulence mechanisms have yet to be established. The practical manufacturing problems of live-attenuated vaccines, as well as the potential to induce HCV infection, restrict their use (287,288).

3.6.2 HCV Genetic variability- A challenge for vaccine development:

The high genetic variability of the HCV virus poses a great obstacle to HCV vaccine development. HCV has 8 genotypes and more than 86 subtypes. HCV strains from various genotypes differ, on average, at around 30% of their amino acids, whereas distinct subtypes within each genotype differ at an average of approximately 15% of their amino acids (289,290). In addition to diversity among genotypes and subtypes, immune selection and the virus's error-prone polymerase generate a diverse quasispecies of related but genetically distinct viral variants within each infected individual, presenting numerous opportunities for selection of viral variants resistant to T-cell and antibody responses (291–294). Given the viral variability inside and between infected people, vaccine-induced highly broadly reactive immune responses or the production of immune responses that target genetically conserved sections of the viral genome may be necessary for protection against HCV infection or persistence.

3.6.3 Challenges for testing HCV Vaccine:

Primary HCV isolates have a restricted capacity to multiply in tissue culture, which limits their production. Furthermore, initial HCV isolates are not produced in good manufacturing process-compliant cell lines and do not reflect the viral variety of the quasispecies circulating in natural infection. Direct infusion of HCV-infected human plasma may be attempted, but it would need rigorous screening for other infections and, even with careful selection of inoculum levels and

HCV genotypes, may fail to fully replicate natural exposure. Suitable animal models were also not available for HCV. Therefore, it can be said that the lack of in vitro techniques and immunocompetent small animal models that help assess whether immunisation develops protective immunity is another barrier to vaccine development. The only animal model that is available for HCV vaccine testing is chimpanzee which is not easily accessible.

3.6.4 T- cell-mediated protection:

Human and chimpanzee investigations have shown that HCV-specific CD4+ and CD8+ T lymphocytes are critical in the suppression of initial and secondary HCV infections. Genetic investigations have revealed a link between HCV clearance and certain class-I and class-II HLAs, which deliver HCV peptides to CD8+ and CD4+ T cells, respectively. This provides indirect support for T-cell control (291,295). A strong and specific proliferative CD4+ T-cell response against HCV proteins has been linked to spontaneous clearance of acute HCV infection (296,297). Successful immunisation requires the generation of an efficient memory response. Reinfection studies support the critical role of T-cell memory in HCV control. Antibody-mediated reduction of CD4+ T cells before reinfection of two immunological chimpanzees resulted in HCV survival despite intact intrahepatic memory of CD8+ T-cell responses (298). Antibody-mediated reduction of CD8+ T cells immediately prior to the third infection of two previously infected chimps resulted in protracted viremia, which was reduced only when CD8+ T cells were detected in the liver.

3.6.5 HCV Vaccines went for trials so far:

Non-structural (NS) proteins (NS3, NS4, and NS5) are more conserved among HCV genotypes than envelope glycoproteins, and they are the primary targets of CD8+ T lymphocytes (299). Various ways have been devised to introduce NS protein antigens in an immunogenic way,

including DNA-based immunisation, recombinant adenovirus priming and DNA boosting, DNA priming followed by recombinant virus vector or HCV protein boosting, combinations of replicating and non-replicating recombinant viruses for prime and boost, virus-like particles (VLPs), and pooled synthetic class I peptide epitopes or peptides incorporated in lysosomes (300–302). Fewer potential vaccinations have been tested for immunogenicity and capacity to protect chimps against HCV. These vaccines include VLP composed of the HCV E1, E2, and core proteins; recombinant non-structural proteins, prepared with the ISCOMATRIX adjuvant; and genetic vaccinations encoding non-structural proteins (300,303–305). However, all these studies were carried out with fewer than 6 animals per study.

Vaccination of chimpanzees with recombinant NS3, NS4, and NS5 proteins synthesised with the ISCOMATRIX adjuvant resulted in sustained HCV infection on rechallenge in all five vaccinated animals, despite the presence of HCV-specific CD4+ and CD8+ T cells in the liver before challenge and infection(306). Chimpanzees were inoculated with DNA plasmids expressing core-E1-E2 and NS3 for priming and recombinant modified vaccinia (MVA) expressing core-E1-E2 and NS3 gene sequences as a boost, resulting in HCV-specific antibody and T-cell responses (307). Despite of protective responses generated by these vaccines; HCV virus persistence remained high in the animals.

The fact that vaccinations have failed even when they elicited T-cell responses suggests that trials should be conducted with caution and incorporate measures for identifying the characteristics of immune responses linked with control. In general, the limited number of chimps examined, the variety of vaccinations tried, and the many methodologies used to measure induced immune responses made it extremely difficult to establish which parts of the immune responses offered protection. It's also uncertain if the findings from nonhuman monkey research can be applied to people. More extensive phenotypic and functional assessments on

existing and future trial specimens are needed to uncover characteristics that influence whether a vaccination reduces the risk of persistent infection in humans.

Chimpanzee Adenovirus vector (ChAd) expressing the NS region of HCV was discovered to produce long-lasting T- and B-cell memory responses in mice and macaques. Adenoviral vectors producing NS proteins from HCV genotype 1b were created using the uncommon serotypes Ad6 and ChAd3. The Ad6-NS and ChAd3-NS vaccines were studied in a safety and immunogenicity phase 1 clinical study in healthy volunteers who were not at risk of HCV infection (308,309). This vaccine lighted hope for the HCV vaccine. Unfortunately, this vaccine also failed in a clinical trial with the PWIDs cohort (clinical trial- NCT01436357) (310). The specific explanation for this result is unknown, however, it may be attributable to the immune-suppressive nature of PWIDs. However, recent epitope-based research indicated a greater immune response in the mouse model than CHAD3-NSmut-1b. This study examined conserved epitopes from diverse HCV genotypes and discovered additional increased responses, including those that were not covered by CHAD3-NSmut-1b (311). Therefore, an epitope-based alternative strategy can be highly effective in vaccine development against HCV, as well as the prediction and confirmation of immunogenic epitopes from HCV. The NS3 region of distinct subtypes is extremely important in vaccine development for HCV.

3.7 Epitope-based vaccine design with immune-informatics approach:

Computational epitope-based vaccine design provides the foundation for modern vaccine development. Epitope-based vaccinations are regarded as a cost- and time-effective technique for developing vaccines with maximum therapeutic efficacy and minimal adverse responses due to the selection of appropriate components [antigens (Ags), epitopes, peptide linkers, and intramolecular adjuvants].

The first epitope-based vaccine was introduced in 1985 (312). This technique involves recognising immunodominant epitopes and inducing immune responses. An epitope is an immunogenic portion of an antigen (Ag) that can be recognised by B cell epitopes (BCEs) or T cell epitopes (TCEs), leading to immune responses (humoral or cellular). T-cell epitopes are usually peptide fragments, but B-cell epitopes may be proteins, lipids, nucleic acids, or carbohydrates (313). Peptides have emerged as promising vaccination candidates due to their relative ease of synthesis and assembly, chemical stability, and lack of infectious potential. Peptide vaccines for different malignancies have been created and reached phase I and phase II clinical trials, with positive clinical outcomes. The peptide vaccine is commonly explored for use in both ameliorating and preventive immunotherapy (314,315).

The immune system only recognises the epitope (part of the antigen), not the full antigen, specifically antibodies, B cells, and T cells. The epitopes may belong to both foreign and self-proteins, and they can be classified as conformational or linear based on their shape and integration with the paratope (316). T-cell epitopes are displayed on the surface of an antigen-presenting cell (APC) and bind to major histocompatibility complex (MHC) molecules to activate immunological response. MHC class I molecules typically present peptides between 8 and 11 amino acids in length, but peptides binding to MHC class II may have a length of 12 to 25 amino acids (317,318).

The rapid development of bioinformatics tools and applications, together with a large amount of experimental data, has had a profound influence on immunology research. This has resulted in a quick rise in the field of computation immunology and a lot of immunology-focused tools and software that aid in understanding the features of the entire immune system have become accessible. Immuno-informatics research focuses primarily on the development and evaluation of algorithms for mapping possible B- and T-cell epitopes, which reduces the time and expense required for laboratory investigation of pathogen gene products. Using such techniques and

information, an immunologist may examine sequence sections with possible binding sites, resulting in the creation of novel vaccines. The approach of analysing the pathogen genome to find possible antigenic proteins is known as “reverse vaccinology (RV)” (319).

There are three major phases in rationalised epitope-based vaccine design: (i) predicting immunogenic portions of antigen using in-silico epitope mapping; (ii) building the immunogenic construct by in-silico docking; and (iii) evaluating the vaccination effectiveness.

The major important factors are-

3.7.1 Antigen selection and optimization:

The correct selection of antigen is a vital stage in creating vaccines, and the new approach of RV permits the scientifically reasoned selection of potential Ags instead of the old procedure of antigen selection. Computational study of pathogen genomes, high-throughput screening, and data-profiling approaches can help identify a suitable antigen (320). To effectively combat an infectious agent, it is possible to target one or many antigens based on their pathogenicity mechanism. ‘Vaxigen’ and ‘vaxDB’ are very useful tools for this purpose (321,322).

3.7.2 T-cell and B-cell epitope prediction:

Traditionally, epitope identification involves sophisticated processes such as Q-TOF and tandem mass spectrometry, hydrogen-deuterium exchange, ELISPOT test, monoclonal Ab-based identification, X-ray co-crystallography, and phage peptide libraries. All these procedures are expensive, but an alternate option for identifying epitope(s) is to employ in silico prediction algorithms, which are both time and cost-effective. Epitope prediction involves analysing antigenic peptides' binding affinity to MHC molecules. The most successful suggested approach is to anticipate TCEs in silico, a data-driven technique is used to predict the peptide-binding properties of certain MHC alleles (class I or class II) (323).

3.7.3 Selection of adjuvant:

The discovery, development, and optimization of effective adjuvants are crucial for improving the efficacy of a planned vaccine. Adjuvants are molecular complexes that, when given as part of a vaccine, boost the immune response. Peptide-based vaccines may naturally result in low immunogenic. Even with the proper BCEs and TCEs, immunogenicity remains low. The intensity and duration of the immune response following vaccination are also important. Thus, Antigens are increased by the choice of an appropriate adjuvant, the addition of which may improve the immune response. Adjuvants in epitope-based vaccinations offer benefits such as extending vaccine memory, expanding the Ab repertoire, renewing the immune system in older patients, and allowing for dose-sparing (324).

3.7.4 Selecting linkers:

Linkers, also known as "spacers," play an important role in the design and engineering of protein vaccines. They have a significant role in the structural stability, interdomain interactions, and functioning of vaccines (325). Spacers connect the three main vaccine components: BCEs, TCEs, and intramolecular adjuvants. The union of functional domains without/with an unsuitable linker might have undesired consequences, such as decreased production, misfolded protein structures, and/or reduced bioactivity (326).

3.7.5 Other factors of fusion vaccine construct:

The integration of both BCEs and TCEs can be performed using suitable spacers. The vaccination sequence is sent to a protein structure prediction algorithm to build a 3D model. The approach analyses predicted domain sequences in the Protein Data Bank (PDB) for structural similarity and generates hits based on four criteria: E-value, alignment length,

identity, and overall score. The 3D model is then checked with molecular docking with TLR protein structures (327).

3.7.6 Success paradigms for epitope-based vaccine:

This study's methodologies have led to the patenting of peptides and their usage in vaccine development. Itoh and Yamada developed a vaccine (US20130164314) with 6-13 peptides produced from tumour antigens, resulting in a wide immune response against malignancies (328). Kudo and Kawami produced a vaccine (US20130122029) that uses two peptides to generate a snail protein, resulting in strong immune responses against malignancies. There were other reports which show that the epitope-based vaccine also was in the trial stage and vaccines were well immunogenic and tolerated (329,330).

Computational approaches for mapping epitopes and designing safe vaccinations have received significant interest. A wide range of databases, web servers, and independent applications are now available for computing and automating various aspects of vaccine design and validation. Epitope-based vaccine design employing in silico computational approaches has been shown to effectively generate immunogenicity without causing cytokine storms or immunological tolerance. Epitope-based vaccines provide benefits over other types of vaccines, including quick and precise design, cost-effective formulations, desirable immunogenicity, and minimal side effects, according to in vitro and in vivo studies.

Hypothesis:

Based on the literature review a hypothesis has been set that if epitopes from HCV proteomes can be predicted and thereby well validated, the dream of HCV vaccination can be achieved. The protein targeted for epitope determination was chosen to be the NS3 protein of HCV. As NS3 protein is an important target for HCV therapeutic intervention, this protein can be selected for epitope determination. Based on the previous studies it was found that HCV genotypes were dependent on geographical factors. HCV is also very versatile which can interfere with therapeutic management. Thereby, regional HCV epidemiology and mutational analysis are necessary, before determining epitopes, so that, we can identify more conserved epitopes which can be used for broad-spectrum vaccine development for the Indian geographical region.

Objectives:

1. Analysis of HCV viremia in different HCV-infected population groups in the eastern part of India and HCV-related disease complications (pathogenesis) among them.
2. Genomic characterization of the isolated HCV strain in different high-risk group populations.
3. Mutational analysis of HCV non-structural gene 3 (NS3) and to find out the important mutations which are associated with chronic HCV and prediction of treatment outcome.
4. To find NS3-host-specific T-cell immune epitopes and their characterization
5. Conservancy analysis and validation of these epitopes.

Workflows:

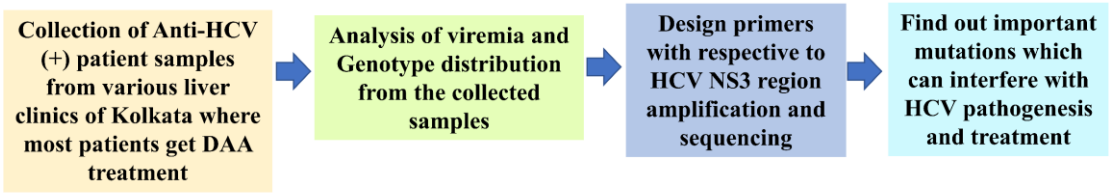

Figure 7: Overall Workflow.

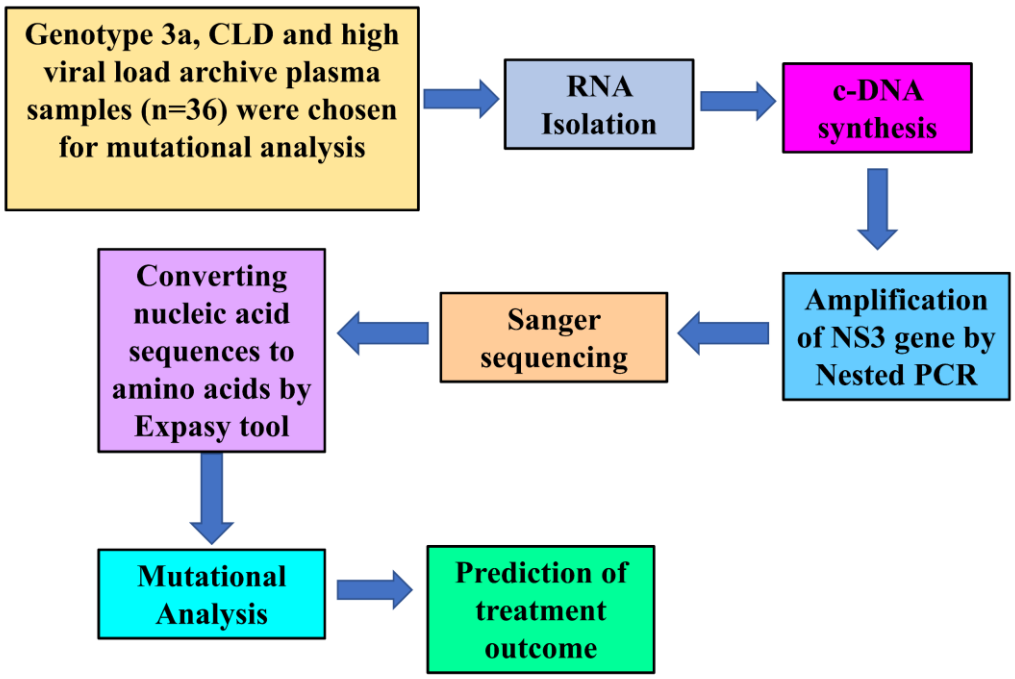

Figure 8: workflow for Mutational analysis of HCV non-structural gene 3 (NS3) and to find out the important mutations which are associated with chronic HCV and prediction of treatment outcome.

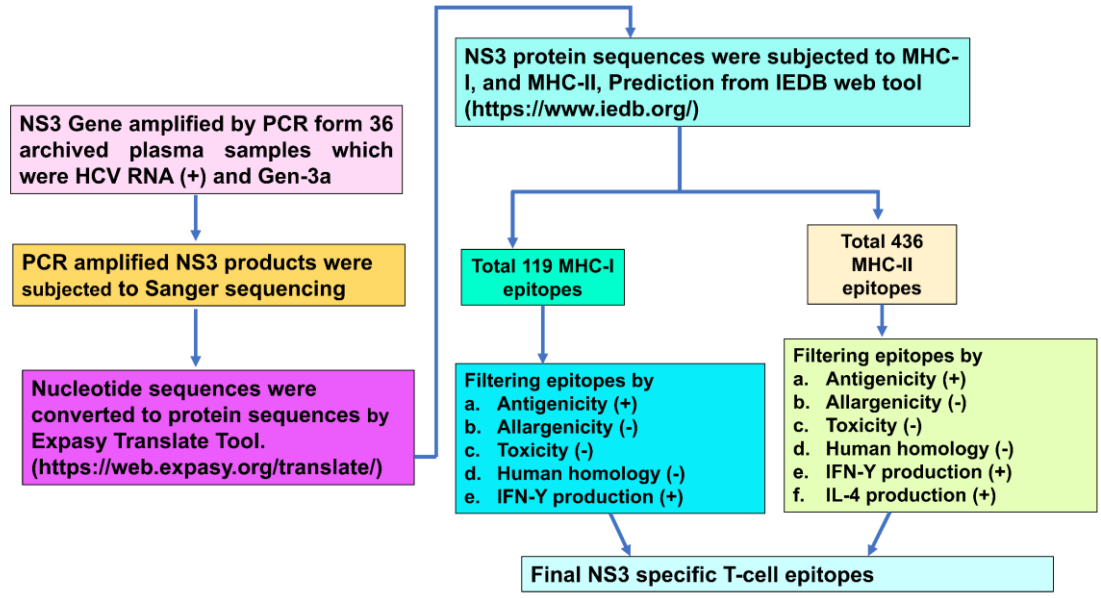

Figure 9: Workflow for finding NS3-host-specific T-cell immune epitopes and their characterization.

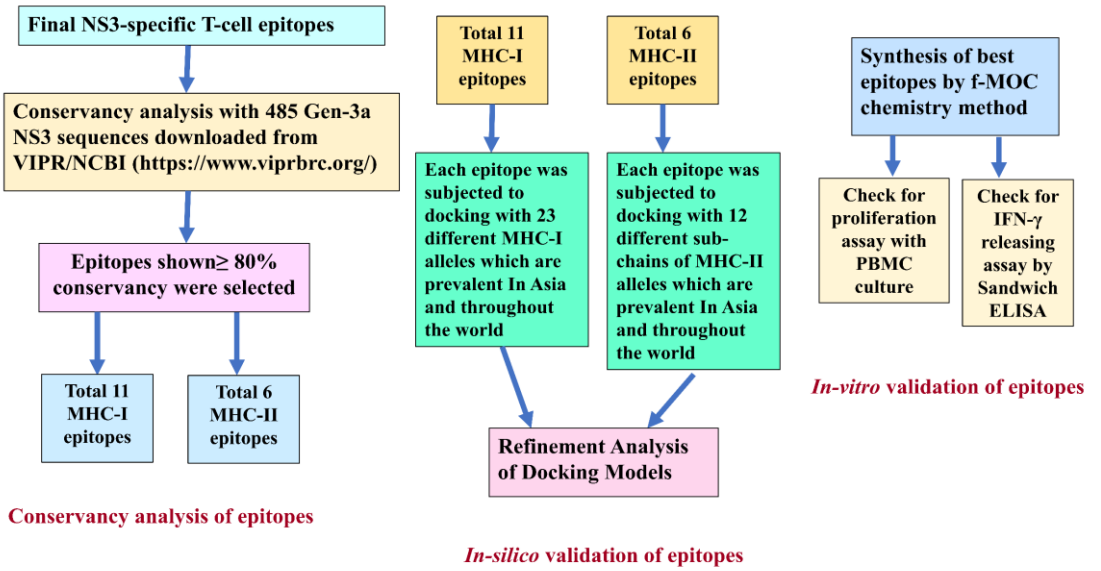

Figure 10: Workflow for conservancy analysis, in-silico, and in-vitro validation of epitopes.

Chapter 4

4 Methodology:

4.1 Population and sample:

4.1.1 Study population and ethical declaration:

In this doctorate study, only HCV seroreactive patients were recruited from different medical colleges and liver clinics in West Bengal, especially in Kolkata (From October 2017 to December 2023). Patients were also sorted based on their clinical risk groups. For example, Thalassemia, Chronic kidney disease, haemophilia, Chronic liver disease, PWIDs etc. belong to the high-risk group population and the general population with chronic liver diseases. Clinical history, test results, blood transfusion history, surgery history or other demographic data were collected in Case Report Form (CRF) from patients at the time of inclusion in a predetermined manner. Patients' histories of drug or alcohol misuse (if any) were also collected during taking consent.

A total of 661 HCV seroreactive patients were enrolled in this study, of which 535 patients belong to high-risk groups, while the rest 126 patients belong to the general population with Chronic Liver Disease (CLD). Patients co-infected with Hepatitis B virus and HIV were excluded from this study.

Informed consents were collected in written format from each patient. If the patients were minors, then consents were collected from their legal guardians. The study protocol followed the ethical principles of the 1975 Helsinki Declaration and was approved by the "Institutional

Ethical Committee" of the "National Institute of Cholera and Enteric Diseases, Indian Council of Medical Research" (Approval number: A-1/2016-IEC dated: 03.10.2016).

All individuals included in this study, voluntarily participated. No blood samples were collected without the patients' consent.

4.1.2 Blood collection and storage of samples:

From each individual, 3 – 5.0 ml blood was collected in an EDTA vial/clot vial by venipuncture method. Blood was drawn by well-trained phlebotomists. Within 2 hours of collecting blood, the samples were delivered to the laboratory via a cold chain for further processing. In the lab, tubes were centrifuged at 1,500g for 5 minutes at room temperature to obtain plasma or serum.

Plasma or serum was separated by a micropipette and aliquoted into three Eppendorf tubes (1.0 ml). Each Eppendorf tube was properly labelled with distinct lab codes and stored in a -80^{o}C deep freezer until further use (gradual freezing applied). Repeated freeze-thaw of samples was always avoided to degrade viral RNA.

4.2 Detection of HCV viremia:

Hepatitis C viral RNA detection provided a measure of active viremia in HCV-infected people. Current NVHCP (National Viral Hepatitis Control Program) guidelines emphasise the necessity of testing HCV RNA levels at baseline before therapy, and at intervals throughout treatment to monitor antiviral response and post-treatment sustain viral response (SVR). A similar approach was used to examine the efficacy of therapy. HCV viremia was detected in the following way:

4.2.1 Viral RNA extraction:

Viral RNA was extracted from HCV seroreactive plasma samples using the QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) following the manufacturer's procedure. In short, 140µl of plasma was taken in a 1.5 ml microcentrifuge tube followed by the addition of 560µl of newly made buffer AVL containing 5.6µL carrier RNA. The microcentrifuge tube was inverted twice to ensure mixing followed by a short spin. The tube was incubated for 10 minutes at room temperature followed by 560µl of 96%–100% ethanol was added and mixed well for 15 seconds by inverting the microcentrifuge tube. The mixture was then loaded into the QIAamp spin mini-column and centrifuged at 6000 x g (8000 rpm) for 1 min. The column was washed with 500µl of wash buffer AW1 followed by a centrifugation at 6000 x g (8000 rpm) for 1 minute. 500µl of wash buffer AW2 was added to the column, and centrifuged at 20,000 x g; (14,000 rpm) for three minutes. The QIAamp-mini column was centrifuged once more at maximum speed for one minute to remove the extra ethanol. The column was then placed into a 1.5ml sterile, nuclease-free microcentrifuge tube. 50µL of elution buffer AVE was then added and incubated for 2 mins, followed by centrifugation at 6000 x g (8000 rpm) for 2 minutes. The extracted viral RNA was aliquated into two batches of 25µl each. The aliquots were stored at -80°C for further use.

4.2.2 Nested RT-PCR amplification to detect HCV viremia:

Based on the 5′ non-coding region (NCR) of the HCV genome, nested PCR was used to detect HCV viral RNA. The primers used in this study were previously used by Bukh *et. al*. (331).

The first round one tube RT-PCR mix (20 µl) was prepared using 4 µl of 5x Go-Taq PCR buffer (Promega, Maddison, USA) 0.4 µl of 10 µM dNTP (Eurogentec, Belgium), 2.5mM of $MgCl_2$, 5mM of dithiothreitol (DTT) (Sigma-Aldrich, USA), 0.25µM of forward and reverse first round primers (PS1 and PA1), 0.4U of AMV reverse transcriptase (Promega, USA), 0.5 U of

Go-Taq DNA polymerase enzyme (Promega, USA) and 3µl of isolated RNA. The RT-PCR cycle protocol was 42°C for 60 minutes followed by 94°C for 5 minutes, followed by 30 cycles

Sl. No.	Name	Primer sequence (5'-3')
1	PS1	ACT GTC TTC ACG CAG AAA GCG TCT AGC CAT
2	PA1	CGA GAC CTC CCG GGG CAC TCG CAA GCA CCC
3	PS2	ACG CAG AAA GCG TCT AGC CAT GGC GTT AGT
4	PA2	TCC CGG GGC ACT CGC AAG CAC CCT ATC AGG
5	HCA1	ATG TAC CCC ATG AGG TCG GC
6	HCS1	ACT GCC TGA TAG GGT GCT TGC
7	HCA2	CAC GTT AGG GTT CGA TGA C
8	HCS2	CAC GTT AGG GTA TCG ATG AC
9	130S	CGG GAG AGC CAT AGT GG
10	290R	AGT ACC ACA AGG CCT TTC G

Table 2: List of Primers used for HCV RNA testing and genotyping.

of 94°C, 55°C and 72°C for 1 minute each, the final extension step was carried out at 72°C for 5 minutes in a Veriti 96 well thermal cycler (Applied Biosystem, USA). 2µl of the first round of RT-PCR amplified product was then subjected to 2nd round nested PCR in a total volume of 25µl containing 5 µl of 5x Go-Taq PCR buffer (Promega, Maddison, USA) 0.8µl of 10µM dNTP (Eurogentec, Belgium), 2.5mM of $MgCl_2$, 0.25µM of forward and reverse second round primers (PS2, and PA2), 0.625U of Go-Taq DNA polymerase enzyme (Promega, USA). PCR cycling condition was 94°C for 5 minutes followed by 30 cycles of 94°C, 55°C and 72°C for 1 minute each, the final extension step was carried out at 72°C for 5 minutes in a Veriti 96 well thermal cycler (Applied biosystem, Massachusetts, USA). The PCR amplicon size was 256 bp. The nested PCR products were analysed in 1.5% agarose gel under a Gel documentation system (Bio-Rad, California, USA). The 256 bp band was ensured of positive HCV viremia.

4.2.3 Quantitative estimation of HCV viral RNA:

HCV viral load was estimated by Superscript III™ platinum™ one-step QRT PCR kit (Invitrogen, Massachusetts, USA). HCV primers from the UTR region (130S, and 290R) and a probe sequence (FAM-CTG CGG AAC CGG TGA GTA CAC- TAMRA) were used for this study. FAM was taken as a fluorophore and TAMRA was taken as a quencher in this study. Estimation was carried out using 4µl of the isolated viral RNA extracted from plasma in total volume of 25 µl containing 12.5µl of 2X PCR Buffer, 1.5µl of detection enhancer, 0.5µl of 10µM forward and reverse primer, 0.5µl of 3µM probe and 1.0µl of 25X Enzyme Mix. The qRT-PCR reaction conditions were 50°C for 15 minutes, followed by 95°C for 2 minutes then 45 cycles of 95°C for 15 seconds and 60°C for 1 minute in a 7500 real-time PCR system (Applied Biosystems, Massachusetts, USA). NIBSC code 06/102, the 4th WHO International Standard for HCV, was used as the standard reference. Log10 international units per millilitre, or log10 IU/mL, was used to represent HCV viral loads.

4.3 Determination of HCV genotype:

It was reported that the HCV core gene was found to be highly conserved. Various studies were also considered the core region for HCV genotyping. Therefore, HCV core-based genotyping was considered for this study.

4.3.1 Amplification of HCV core gene:

Partial core (structural) sequences were amplified using nested RT-PCR for HCV genotyping. For 1st round PCR, the outside sense primer HCS1 and the outer antisense primer HCA1 were utilized, and the inner sense primer HCS2 and the inner antisense primer HCA2 for 2nd round PCR.

For 1st Round and 2nd rounds of amplification master mix-making and thermal cycling stage remained the same as mentioned above except the primers used (for 5′ UTR amplification), instead of PS1 and PA1, HCS1 and HCA1 were used in 1st round and instead of PS2 and PA2, HCA2 and HCS2 were used in 2nd round.

12µL of final PCR amplicons (405 bp) were analysed with 1.5% agarose gel electrophoresis followed by a Gel doc system (BioRad, USA). The DNA fragments (bands) were cut by a sterile scalpel and stored into properly labelled corresponding sterile Eppendorf tubes (1.5ml) and stored in a -20°C freezer for gel cleanup.

The rest of the amplified products were then transferred to a -20°C freezer until further use.

4.3.2 Purification of PCR amplified product:

For sequencing, the positive amplicons were subjected to gel purification. The Nucleospin Gel and PCR clean-up kits (Macherey-Nagel, Germany) were used for this purpose. Gel purification was done as per the manufacturer's recommended protocol. Briefly, the gel-cut PCR products were mixed with 200µl of NT1 buffer and heated at 60°C in a thermomixer for 5 minutes (Model- Thermomixer Comfort, Eppendorf, Germany). When the gel was fully dissolved, the mixture was added to the PCR cleanup column followed by a centrifugation at 11000xg for 30 seconds. The spin column was then washed twice with 700µl NT3 buffer followed by a spin at 11000xg for 1 minute to dry the column. Spin columns were then air-dried for 2 minutes to ensure that traces of alcohol were removed. Previously heated 50µl elution buffer (NE) was then added to spin columns and centrifuged at 11000×g for 2 minutes to get purified DNA. These purified DNA were then stored in a -20°C freezer for sequencing.

4.3.3 DNA sequencing by Sanger method:

Sequencing was performed with 405bp core amplified products. The Big Dye Terminator 3.1 cycle sequencing kit (Applied Biosystems, Massachusetts, USA) was used for Sanger sequencing using both forward and reverse primers (HCS2 and HCA2). The Genetic analyser 3130xl (Applied Biosystems, Massachusetts, USA) was used for this purpose.

Briefly, a master mix using the following protocol for the chain termination reaction setup. 10µl of master mix for sequencing PCR was prepared with 1.75µl of 5x sequencing buffer (Applied Biosystem, USA), 1µl of HCA2 or HCS2 (0.8 µM), 0.7µl of BigDye terminator, 4.55µl of nuclease-free water and 1µl of gel purified DNA. Big Dye standardised PCR cycling was carried out according to the manufacturer-recommended protocol.

Following the BigDye Terminator sequencing reaction, excess labelled nucleotides (which can obstruct early sequence data and cause base calling issues) were eliminated using the ethanol/EDTA/sodium acetate precipitation method. Briefly, two master mixes were made:

1. For each reaction, 10µl of miliQ water and 2µl of 125mM EDTA were combined to create Master Mix-1.
2. Mixture Master-2 was made with 2µl 3M NaOAc (Sodium Acetate) 50µl of ethanol per reaction was produced at a pH of 4.6.

Each reaction contains 10µl of sequence reaction and 12µl of master mix1. The contents were mildly mixed followed by the addition of 52µl of Master Mix 2. The entire mixture was incubated for fifteen minutes at room temperature. The supernatant was aspirated after centrifugation at 12,000xg for 20 minutes. 500µl of 70% ethanol was added to the pellet at room temperature to wash it, and centrifuged again for 20 minutes at 12000xg. The washing process was repeated once more with 70% ethanol and the supernatant was aspirated. The pellet was air-dried to remove traces of ethanol and dissolved in 15µl of formamide. The dissolved

products were heated at 95˚C followed by a quick chilling in ice. The mixture was briefly vortexed and loaded directly into the genetic analyser for sequencing.

4.3.4 Sequence data editing and determination of genotype:

The PCR-amplified product was sequenced from both the forward and reverse ends. Electropherogram sequencing data was edited with Bio-Edit software, version 7.2 (https://bioedit.software.informer.com/). All unambiguous bases were deleted using Bio-Edit software and sequences were retrieved in '. fasta' format.

For HCV genotype determination NCBI genotyping tool (https://www.ncbi.nlm.nih.gov/projects/genotyping/help.html) was used. HCV partial core (405bp) sequences were uploaded in '.fasta' format in the NCBI genotyping tool which searches for similarities with other reference HCV genotypes and generates a similarity window with scores based on 300bp window length. Genotype was determined by the highest similarity matching score generated by the genotyping tool.

4.4 Phylogenetic and phylogeographic analysis:

Molecular Evolutionary Genetics Analysis software (MEGA version X) was used to build a representative phylogenetic tree to examine the evolutionary relationships between the HCV sequences that were taken from the study population. Using various reference sequences of different HCV genotypes and subtypes downloaded from NCBI, the first 300 bp (similar to 371nt to 671nt of the H77-accession number NC-004102) of the partial-core gene sequence was aligned using the CLUSTAL OMEGA programme in MEGA-X (332). The Tamura-Nei (TN) parameter model was the best-fit model for building the evolutionary tree, and the maximum likelihood approach was employed beneath it (333).

The geographic origin and dispersion of HCV infections in and from West Bengal were identified using phylogeographic analysis, which was utilized to estimate viral migratory

events. The software Beast 1.10.4 (334)was used to construct a phylogeography tree of the partial HCV core gene of genotypes 3a, 3b, 1a, 1b, and 4a. Using the CLUSTAL OMEGA tool in the MEGA-X software, reference sequences from various countries retrieved from the NCBI, VIPR, and Los Alamos HCV databases were aligned with the reported sequences. Model testing was also performed in MEGA-X and the obtained best-fit model Hasegawa–Kishono–Yano (HKY) + gamma was used to construct the phylogeography tree (31). The Bayesian Skyline Coalescent model (335) and the uncorrelated relaxed clock type was employed for the clock setup. Path Sampling (PS)/stepping-stone (SS) analysis was used to undertake Markov Chain Monte Carlo (MCMC) studies (31). In TRACER (http://beast.community/tracer), Effective Sampling Sizes (ESS) were achieved by running MCMC for 200 million states and sampling every 10,000 states. TRACER was used to analyse the BEAST analysis findings(336). ESS value, generated by TRACER, greater than 200 denotes good sampling. The final tree was built using the program Tree Annotator (https://beast.community/treeannotator) with the burn-in value generated by TRACER and the Figtree (https://beast.community/figtree) program was used to visualize the final tree.

4.5 Mutational analysis of HCV NS3 gene:

HCV genotype 3a was found to be the most prevalent one in this region. So, we have chosen this genotype for this analysis. A total of 36 HCV genotype 3a RNA samples were selected. All clinical histories of these patients were collected from collaborative Liver Clinics. These samples were chosen such that they did not have any history of co-infections.

4.5.1 Designing of NS3 specific primer for HCV gen-3a strain amplification:

In-house NS3-specific primers were designed for the amplification of the complete NS3 gene from the genotype 3a strain circulated in this region. For that, first HCV 3a full genome sequences were retrieved from the BVBRC website. The full genome sequences were chosen with a filter option; specific regions (positions) were marked with annotation with the data found from benchling. Finally designed primers were checked with BLASTn for final conformation. Virtual PCR was performed to ensure proper annealing and length measurement. The primers found by this analysis (**Table 3**) are as follows:

Sl. No.	Name	Primer sequence (5'-3')
1.	NS3OUF	GGG CCG CAG CCG GTC TCA AAG ACC
2.	NS3OUR	GTT CGA TGT ATG GGG CGG CCT GCG
3.	NS3INF	GCT CCG ATC ACA GCA TAC GCC CAG C
4.	NS3INR	GGTGGTTACTTCCAGGTCGGCTGACATGC

Table 3: List of Primers for amplification of complete NS3 gene

4.5.2 Amplification of NS3 gene (HCV genotype 3a) and sequencing:

A total of 36 HCV RNA samples that were not co-infected with HBV or HIV or any other viruses were used for complete HCV NS3 gene amplification. cDNA was synthesised using superscript III reverse transcriptase (Invitrogen, CA, USA) according to the manufacturer's

recommended protocol. Outer reverse primer (NS3OUR) was used for the cDNA synthesis. The NS3 region was amplified with nested PCR using the Expand^{TM} long template PCR system (Sigma Aldrich, USA). For 1st round PCR, a master mix (23µl) was prepared with 2.5µl of 10x PCR buffer ($MgCl_2$ included in the buffer), 1µM of outer forward (NS3OUF) and outer reverse (NS3OUR) primer, 1µM of dNTP, and 3.75U of expand long template DNA polymerase mix (Sigma Aldrich, USA). 2µl of cDNA was added to the master mix and the PCR was performed in Veriti 96 well thermal cycler (Applied Biosystems, Massachusetts USA). The PCR temperature cycling protocol was as follows: 94ºC for 2 min (denaturation); 10 cycles with 10s denaturation at 94ºC, 30s annealing at 60ºC, and 2 min extension at 68ºC; 25 cycles with 10s at 94ºC, 30s at 60ºC and 2min (with +20s in each cycle) at 68ºC; and 68ºC for 7min (final extension) followed by 4 ºC for storage. 1st round and 2nd round PCR protocols were the same except for the use of the primers. In 2nd round PCR, 2µl of the first-round PCR product (1: 100 dilution) was used. Two primers for 2nd round PCR, inner forward NS3INF and inner reverse NS3INR were used. Enzymes, protocols and cycling conditions were the same as in the first round. Agarose gel electrophoresis was performed for the nested PCR amplified products in a 1% agarose gel, a 1893bp PCR positive bands were then cut and purified with Nucleospin Gel

Sl. No.	Name	Sequence (5'-3')
1.	3aNS3seqFa'	CCTCTGGAGGTCCTGTTATGTGCCCTTCG
2.	3aNS3SeqRa'	ACCGTCAGCCTCACCCCAGCC
3.	3a NS3seqRa"	CCGAAGGGCACATAACAGGACCTCCAGAG
4.	3aNS3SeqFb'	GGCTGGGGTGAGGCTGACGGT
5.	3aNS3seqRb'	GAGCAGCCCGCGTCATAGCACTCAC
6.	3a NS3seqFc"	GTGAGTGCTATGACGCGGGCTGCTC

Table 4: List of Primers for sequencing of complete NS3 gene.

and PCR purification kit (Macherey-Nagel, Germany) with the above-mentioned protocol in 6.3.2.

Purified products were then processed for the Sanger sequencing along with primer walking. The BigDye™ Terminator 3.1 cycle sequencing kit (Applied Biosystems, USA) was used for automated di-deoxy nucleotide termination sequencing. A total of six primers (**Table** 4) along with NS3INF and NS3INR were used for the whole 1893bp sequencing. Other protocols were the same as 6.3.3.

Electropherogram Data were analysed (viewing, editing and contig joining) by Bio-Edit software (https://bioedit.software.informer.com/). All sequences were reported to NCBI for accession numbers.

4.5.3 Translation of NS3 sequences and mutational analysis:

The online Expasy Translate tool (https://web.expasy.org/translate/) was used to translate reported HCV NS3 nucleotide sequences into protein sequences. The Esprit 3 webpage (https://espript.ibcp.fr/ESPript/ESPript/) and MEGA-X provided protein sequence alignment. For detailed mutational analysis, the Bio-Aider tool (https://bio.tools/bioaider) was used (337).

4.6 Prediction of conserved NS3-specific T-cell epitopes:

All NS3-specific amino acid sequences found in 6.6.3 were subjected to the consensus maker tool (http://kirr.dyndns.org/bio/consensus/). A consensus sequence was generated by the consensus maker tool.

4.6.1 Prediction of MHC-I and MHC-II epitopes:

The MHC-I and MHC-II epitope prediction was carried out using the consensus HCV NS3 protein sequence. In this study, the Immune Epitope Database (IEDB) server (https://www.iedb.org/) was used. The National Institute of Allergy and Infectious Diseases (NIAID) oversees maintaining this database and a free public database. The default parameter given by the server was used to anticipate the MHC-I and MHC-II epitopes. The percentile rank, < 0.5 was used to forecast epitopes; a lower percentile rank indicates a higher projected epitope binding affinity. Additionally, an attempt was made to identify the epitopes that are comparatively conserved throughout the 36 protein sequences.

4.6.2 Filtering MHC-I and MHC-II epitopes:

The fundamental five characteristics were taken into consideration while filtering all MHC-I and MHC-II epitopes:

Antigenicity (estimated using the VaxiJen 2.0 server; http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html]).

The AllerTop 2.0 server's prediction of allergenicity [https://www.ddg-pharmfac.net/AllerTOP/index.html].

ToxinPred (https://webs.iiitd.edu.in/raghava/toxinpred/multi submit.php) predicts toxicity.

IFN-γ production was anticipated using the IFN epitope server [https://webs.iiitd.edu.in/raghava/ifnepitope/predict.php],

human homology was predicted by E value analysis using NCBI-BLASTp [https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins]).

Two extra filtering factors were applied just to MHC-II epitopes:

IL4 production (estimated using the IL4pred server [https://webs.iiitd.edu.in/raghava/il4pred/design.php]).

The IL10 pred server [https://webs.iiitd.edu.in/raghava/il10pred/predict3.php] predicts the production of IL10.

The filtered epitopes selected were further examined for antigenic, non-allergic, non-toxic, non-human homolog (E-value>2), and able to produce IFN-γ. Regarding MHC-II, epitopes underwent further filtration to ensure their potential to produce IL4 and IL10 (338–340).

4.6.3 Conservancy analysis of filtered MHC-I and MHC-II epitopes:

The MHC-I and MHC-II filtered epitopes were further examined via conservancy analysis. To determine the degree of epitope sequence conservation among the different sequences included in the NCBI database, conservation analysis was performed. The HCV (subtype-3a) NS3 protein sequences obtained were undergone for conservancy analysis online from the Bacterial and Viral Bioinformatics Resource Centre (BV-BRC) website. This website was formerly known as the Virus Pathogen Database and Analysis Resource (ViPR). Sequences with issues (X-coded uncertain amino acids) were not subjected to analysis. MEGA-X software was used to obtain and align 485 NS3 protein sequences. The alignment was then subjected to IEDB's epitope conservancy analysis tool [http://tools.iedb.org/conservancy/] , along with epitope sequences for conservancy analysis. For further docking analysis, only epitopes exhibiting > 80% conservation were considered.

4.7 Validation of Epitopes:

4.7.1 *In-silico* validation:

4.7.1.1 Molecular docking and refinement analysis of MHC-I and MHC-II epitopes:

Subsequently, highly conserved epitopes were examined for molecular docking and optimization. Every MHC-I epitope was docked with 23 distinct MHC-I allele types, which are common in the Asian population, and every MHC-II epitope was docked with 12 distinct MHC-II sub-chains (prevalent in the Asian population). The Allele Frequency Net Database [http://www.allelefrequencies.net/] provided information on allelic prevalence. From RCSB PDB, the structures of twelve distinct MHC-II sub-chains and twenty-three distinct MHC-I sub-chains were obtained. With the use of the Discovery studio programme [https://discover.3ds.com/discovery-studio-visualizer-download], these structures were then processed for docking by eliminating ligands, water molecules, other compounds, and duplicate residues or alleles. The Modeller 10.3 programme was used [https://salilab.org/modeller/] to model missing loops. Cleaned (.pdb) structures and (fasta) epitope sequences were analysed for protein-peptide docking using interaction similarity. Every sub-chain for MHC-II molecules was docked independently with MHC-II epitopes. GalaxyPepDock Server [https://galaxy.seoklab.org/cgi-bin/help.cgi?key=METHOD&type=PEPDOCK] conducted the analysis. Results were visualized in Discovery Studio Software and the models which have the highest template modelling score (TM-score) were refined with GalaxyRefineComplex server [https://galaxy.seoklab.org/cgi-bin/submit.cgi?type=COMPLEX] for further refinement and RMSD calculation.

The chosen 23 different MHC-I alleles and their respective PDB IDS were HLA_A_01_01 (6AT9), HLA_A_02_01(4U6Y), HLA_A_02_06(3OXR), HLA_A_02_07(3OXS),

HLA_A_03_01(3RL1), HLA_A_11_01(7S8S), HLA_A_24_02(7JYV), HLA_A_68_01(6PBH), HLA_B_07_02(6AT5), HLA_B_14_02(3BXL), HLA_B_15_01(5TXS), HLA_B_15_02(6VB2), HLA_B_18_01(4XXC), HLA_B_35_01(1A1N), HLA_B_35_08(3BWA), HLA_B_40_01(6IEX), HLA_B_40_02(5IEH), HLA_B_44_03(3DX7), HLA_B_44_05(6MTL), HLA_B_51_01(1E27), HLA_B_52_02(3W39), HLA_B_57_01(6BXP), and HLA_B_58_01(5VWH).

The chosen 12 different sub chains of MHC_II alleles and their respective PDB IDS were HLA_DQ1(1S9V), HLA_DQB(1S9V), HLA_DMA(2BC4), HLA_DMB(2BC4), HLA_DQA1(5KSV), HLA_DQB1(5KSV), HLA_DRA1(3C5J), HLA_DRB3(3C5J), HLA_DPB1(3WEX), HLA_DPA1(3WEX), HLA_DRB5(1H15), and HLA_DRB1(SV4M).

4.7.2 *In-vitro* Validation:

4.7.2.1 Synthesis of peptides (epitopes):

The solid phase peptide synthesis along with the Fmoc chemistry-based approach was used to synthesize the best-scoring epitopes. For this process, Rink amide MBHA resin was used (Sigma-Aldrich, Novabiochem, Germany). Standard amino acids with Fmoc protection were obtained from Sigma-Aldrich and Aapptec (Louisville, USA). Peptides were separated from the resin after synthesis by vigorously shaking in a cleave solution (95% trifluoroacetic acid + 2.5 %t triethyl silane + 2.5 % ethanol) overnight. Finally, Diethyl ether (Spectrochem, India) was used to precipitate the resultant peptides. After that, these peptides were taken in a 15ml centrifuge tube and a brief centrifugation was done. Following an air-drying process, the peptides were resuspended in 30% acetonitrile (Spectrochem, India) and subjected to a high-performance liquid chromatography (HPLC) method to ensure a final purity of higher than 95%. Synthesised peptides (epitopes) were kept at -20^{o} C until used in other experiments.

4.7.2.2 Isolation of Peripheral Blood Mononuclear Cells:

Peripheral blood mononuclear cells (PBMCs) were isolated from ten healthy individuals who had previously been exposed to HCV and had completed the DAAs therapy. In short, venipuncture was used to obtain 5 ml of blood from each person, in EDTA vials. Using the density gradient centrifugation technique and HiSEP^{TM} LSM 1077 (Himedia, India), peripheral blood mononuclear cells (PBMC) were extracted from the blood. In a 15ml centrifuge tube, 3ml of LSM solution was carefully layered over 2:1 PBS diluted blood, and the tube was centrifuged at 500 x g for 45 minutes. The lymphocyte-monocyte layer was carefully isolated in a fresh sterile 15ml centrifuge tube. The lymphocyte-monocyte layer (PBMC) was washed twice with the wash solution (90% PBS and 10% FBS). Finally, PBMC was combined with the freeze-mix solution (90 % FBS and 10 % DMSO) and kept at -80ºC for further use.

4.7.2.3 T-cell Culture:

Lymphocytes were cultured for 7 days according to the method of Lefort & Kim, and Raulf, 2019. Briefly, the PBMCs were transferred to a T-75 culture flask in 20ml of RPMI 1640 medium containing 10% FBS, 1% penicillin/streptomycin, and 1µg/mL phytohemagglutinin (PHA), and incubated at 37°C with 5% CO_2 for maximum 24 hours. This enables the separation of the lymphocytes from the monocytes that stick to the flask surface. Carefully the media containing lymphocytes were collected in a 50ml conical tube and centrifuged for 5 minutes at 500 x g. Cell pellets were washed twice with 90% PBS+ 10% FBS. Thereafter, cells were transferred to a new T-75 flask containing 25ml of RPMI 1640 medium with 10% FBS, 1% penicillin/streptomycin, and 20ng/mL human IL-2. The cells were then pulsed with 1µg/ml PHA and incubated for 7 days.

4.7.2.4 CFDA proliferation assay:

Following a week of lymphocyte culture, about 5 x 10^6 cells were resuspended in 1ml of PBS with 5µM of carboxyfluorescein diacetate succinimidyl ester, or CFDA-SE (Invitrogen, USA). CFDA-SE dye was used as a proliferation marker. CFDA-SE dye is easily permeable to cells where it was cleaved by intracellular esterase to give rise to fluorescent carboxyfluorescein succinimidyl ester (CFSE) compound. CFSE reacts with intracellular amines and establishes a stable, covalent bond that is retained by the cell. With every further cell cycle, the fluorescence intensity of CFSE is almost cut in half. The fluorescence intensity of CFSE is roughly halved with each subsequent cell division. The CFDA-SE dye is slightly toxic for cells. Therefore, in this study, the concentration of CFDA-SE was previously titrated to ensure that the cell toxicity remained in a safe level. Cells were incubated for 15 minutes at 37ºC in PBS+CFDA-SE. Following incubation, cell labelling was quenched for 5 minutes with ice-cold 2ml of PBS+10% FBS, followed by two washes with 5ml of PBS+10% FBS. Washed cells were dissolved in RPMI 1640 supplemented with glutamine to achieve a final density of 3 x 10^6 cells/ml. For the proliferation assay, cells were plated in 24 well plates (duplicated) with 2 x 10^5 cells per well. Each testing well was pulsed with 20µg/ml peptides (epitopes). A negative control was set with no peptide and merely PBS in place of peptides. Positive control was established using phytohemagglutinin (PHA-L, Invitrogen, Brazil). The plate was incubated at 37°C for 7 days. After 7 days, cells were fixed according to the manufacturer's protocol (CFDA-SE, Invitrogen, USA) and analysed using BD FACSAria II (USA) for cytometric analysis. Briefly, the fluorescence of 10,000 lymphocytes was gated according to scatter parameters such as size and granularity. The FACS machine yielded the mean fluorescence value (MFI) for each well. MFI values were then plotted in R (using the ‘ggplot’ package) and compared with non-parametric statistical tests.

4.7.2.5 IFN-γ releasing assay by ELISA:

The IFN-γ ELISA kit (Elabscience, USA) was used to detect IFN-γ release by T cells. Briefly, lymphocytes were cultured in 24 well plates in duplicates and pulsed with epitopes as described earlier in section 6.7.2.4. After 5 days, 200µl of supernatant were taken and tested for IFN-γ levels. The kit recommended procedure was followed to measure IFN-γ level in cell soup. Briefly, cell culture supernatants were added into each well of antibody-coated 96 well plates (provided with the kit). Kit provided positive control was used in this study. As a negative control, PBS-pulsed cell supernatant was used. After loading cell soups to 96 well plates, the plates were sealed and incubated for 90 minutes. After that 100µl of Biotinylated antibody solution was added to each well followed by an incubation at 37°C for 1 hour. Then, wells were washed with 350µl of wash buffer. After that wells were treated with HRP conjugate solution followed by substrate solution and stop solution. Optical density (OD) was measured for each well with a microplate reader (Wavelength Set to 450nm).

Chapter 5

5 Results:

5.1 Analysis of HCV viremia in different HCV-infected population groups in the eastern part of India:

This doctoral study was conducted from September 2017 to December 2023, a total of 661 anti-HCV-positive patients were enrolled and blood samples were collected from each individual. Out of 661 individuals, 250 belong to the thalassemia population, 65 belong to the PWIDs population, 155 individuals belong to the chronic kidney disease (CKD), 40 belong to

Individual Category	HCV Sero-reactive samples (N=661)	HCV RNA (+Ve) (n=403)	% of HCV RNA (+Ve) [60.96%]
Thalassemia	250	163	65.20
PWIDs	65	46	70.70
Hemodialysis/CKD	155	78	50.32
Hemophilia	40	25	62.50
HIV with HCV	25	15	60.00
General Population with CLD (Chronic liver Disease)	126	74	58.73

Table 5: HCV viremia among various population groups in this study.

the haemophilia group, 25 individuals have a history of HIV-HCV co-infection and the remaining 126 belong to the general population with chronic liver disease. All seroreactive blood samples were tested for HCV viremia. Out of the total of 661 samples, 403 samples

were RNA-positive. The highest number of individuals with viremia was observed within the PWIDs group (70.70%), followed by Thalassemia (65.21%) and haemophilia (62.50%). In the CKD population, viremia was found in about 50.32%. Therefore, it can be inferred that overall viremia was 60.96%. The major population group found in this study was thalassemia, followed by CKD, and the general population with CLD. (**Table 5**)

5.1.1 Viremia in thalassemia group:

A total of 250 Thalassemia patients with history of blood transfusion were enrolled in this study. Out of these 250 (146 males and 104 females), 163 (103 males and 60 females) were found to be HCV RNA-positive (65.20%). Most of the thalassemia patients were teenagers. 22.80% of thalassemia patients belong to the age group 1 to 5 years, 25.20% of thalassemia patients fell within the age group 6 to 10 years, 34% of thalassemia patients fell within the age group 11 to 15 years, 9.60% of thalassemia patients were lying within the age group 16 to 20 years, 4.8% of thalassemia patients fell into age group 21 to 25 years, only 2% of thalassemia patients fell within 26 to 30 years and rest 1.6% of thalassemia patients had greater than 30 years of age. This study showed that the thalassemia group had a high risk of HCV infection from an early age. Age group 6 to 10 were found to have the highest viremia in the thalassemia

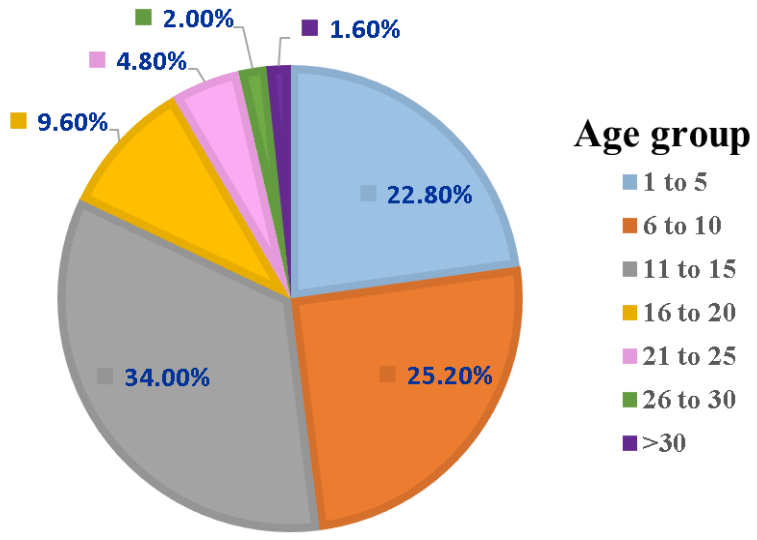

Figure 11: Age distribution among thalassemia patients in this study.

population. It was also found that the risk of HCV in thalassemia is higher in economically weaker people and underdeveloped areas. 76.80% of thalassemia patients in this study belonged to rural areas and 78.80% were from lower economic backgrounds. Whereas, only

21.20% of thalassemia patients in this study belonged to those above the poverty level. The reason behind this may be rural and poor people are unaware of thalassemia disease and HCV infection. The finding suggests that HCV- and thalassemia-related awareness campaigns are needed to overcome this situation (**Table 6**).

Variables		HCV (+) n= 163	HCV (-) n= 87	Viremia (%) 65.20 %	p-Value
Gender	**Male**	103	43	70.54	0.03*
	Female	60	44	57.69	
Age-group	**1 to 5**	38	19	66.67	0.03*
	6 to 10	53	10	84.13	
	11 to 15	49	36	57.65	
	16 to 20	13	11	54.17	
	21 to 25	7	5	58.33	
	26 to 30	3	2	60.00	
	>30	2	2	50.00	
Locality of residence	**Rural**	133	59	69.27	0.04*
	Urban	32	26	55.17	
Economic class	**Below poverty level**	137	60	69.54	0.02*
	Above poverty level	28	25	52.83	

Table 6: HCV viremia with various demographic factors in the thalassemia population in this study.

****Statistically significant.***

5.1.2 Viremia in CKD group:

Overall, 155 anti-HCV (+) patients were found with chronic kidney disease. Among them 99 were male and 56 were female. Out of 155 anti-HCV (+) samples 78 were RNA positive (50 male and 28 female). This study revealed that Most of the CKD patients were above the age of 25 years. It was found that only 11.61% of CKD patients in this study belonged to the age group of 14 to 24 years. 25.81% of the CKD patients in this study, came under the age bracket of 25 to 34 years. 23.87% of the CKD patients in this study belonged to the age group of 35 to 44 years. 21.29% of the CKD patients in this study fell into the age group of 45 to 54 years. 13.25% CKD study population fell into the age group of 55 to 64 years and 3.87% of CKD

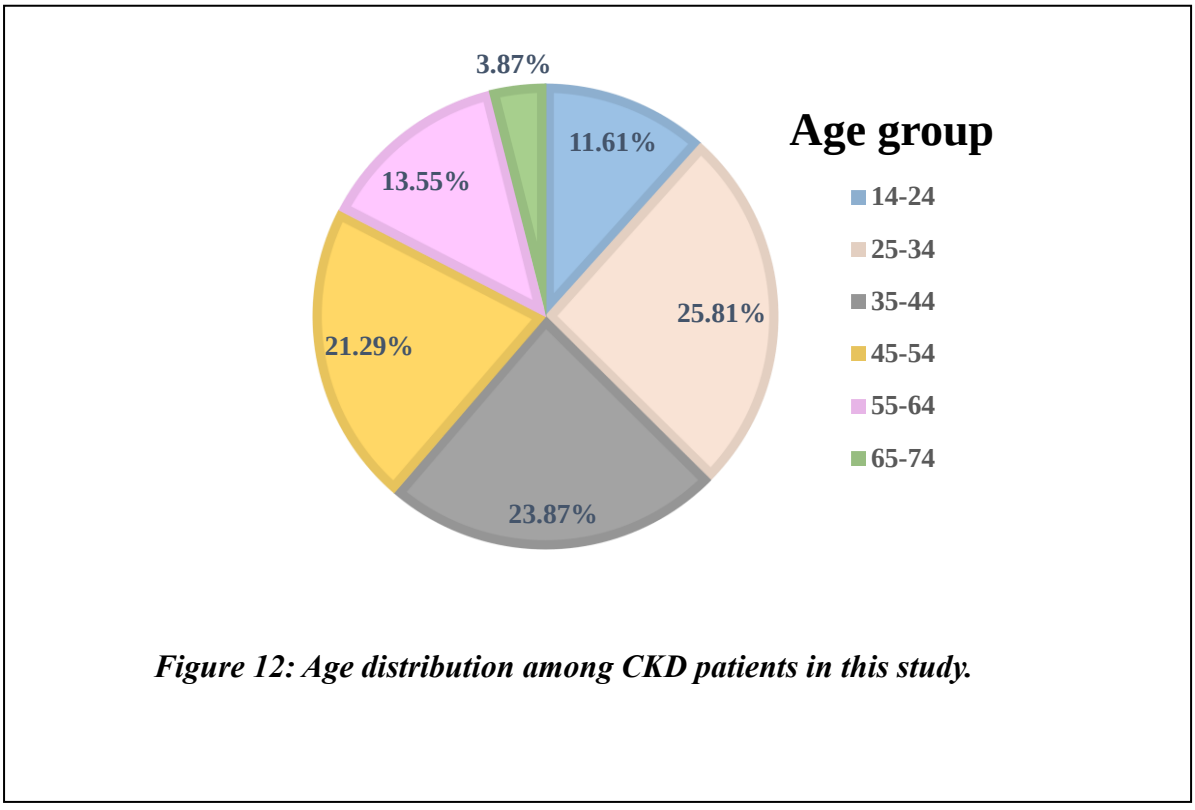

Figure 12: Age distribution among CKD patients in this study.

patients in this study were above the age of 65 years. CKD Patients after middle age were at higher risk for HCV infection. It was also found that with increasing age CKD patients were at higher risk of HCV infection. Specifically, CKD patients in the age group 55-64 were more prone to HCV infection (p-value: 0.01). It was found that CKD patients, who lie between 55 and 64 years, have a chance of HCV infection of about 71%. The risk of HCV infection also

increases with the frequency of dialysis taken per month (p-value: 0.04). The chances of HCV infection increase up to 59% if a patient takes dialysis 3 to 4 times per week. It is also higher in CKD patients reside urban and sub-urban areas (**Table 7**).

Variables (n=155)		HCV (+) (n=78)	HCV (-) (n=77)	Viremia (%) (50.32)	p-Value
Gender	**Male**	50	49	50.50	0.95
	Female	28	28	50	
Age group (year)	**14-24**	6	12	33.33	0.01*
	25-34	13	27	32.50	
	35-44	19	18	51.35	
	45-54	21	12	63.64	
	55-64	15	6	71.43	
	65-74	4	2	66.67	
Dialysis interval	**2 times/month**	6	15	28.57	0.04*
	4 times /month	11	18	37.93	
	8 times/month	48	35	57.83	
	12 times/month	13	9	59.09	
Locality of residence	**Rural**	24	40	37.50	0.02*
	Sub-Urban	21	15	58.33	
	Urban	33	22	60.00	

Table 7: HCV viremia with various demographic factors in the CKD population in this study.

****Statistically significant.***

5.1.3 Viremia in CLD patients:

A total of 126 anti-HCV positive samples were collected who have a history of chronic liver disease. Among them 66 were males and 60 were females. Out of 126 HCV sero-reactive blood

N=126 Variables			RNA (+) n=74	RNA (-) n=52	Viremia (%) 58.73%	p-value
Gender		Male	36	30	54.54	0.31
		Female	38	22	63.33	
Age group		20-27	2	3	40.00	0.87
		28-35	4	3	57.14	
		36-43	9	9	50.00	
		44-51	15	11	57.69	
		52-59	22	12	64.71	
		60-67	18	10	64.29	
		68-75	4	4	50.00	
		76-83	2	3	40.00	
Mode of transmission		Blood transfusion	26	8	76.47	0.04*
		Medical surgeries	16	14	53.33	
		Needles/Syringes	32	30	51.61	
Status of Cirrhosis	Cirrhosis(n=49)	Decompensated cirrhosis	25	7	78.13	0.02*
		Compensated cirrhosis	10	7	58.82	
	No cirrhosis (n=77)		39	38	34	

Table 8: HCV viremia and demographic details of the CLD population in this study.

**** Statistically significant.***

samples, 74 were RNA-positive (36 males and 38 females). Therefore, it can be said that HCV viremia among the CLD population is about 58.73%. It was found in this study pluralities of CLD patients were of 20-27 years (3.82%), followed by 28-35 years (5.34%), 28-35 years (5.34%), 36-43 years (13.74%), 44-51 years (19.85%), 52-59 years (29.25%), 60-67 years (21.37%), 68-75 years (6.11%) and 76-83 years (3.82%). It was also found that the CLD population with age group 52-67, were the highest viraemic (64.29% to 64.71%) among all other age groups although statistical significance was not observed. It was seen that CLD patients who had a history of blood transfusion were more vulnerable towards HCV infections (p-value- 0.04). Decompensated cirrhosis of liver patients also showed higher viraemia compared to other groups (p-value- 0.02) (**Table 8**).

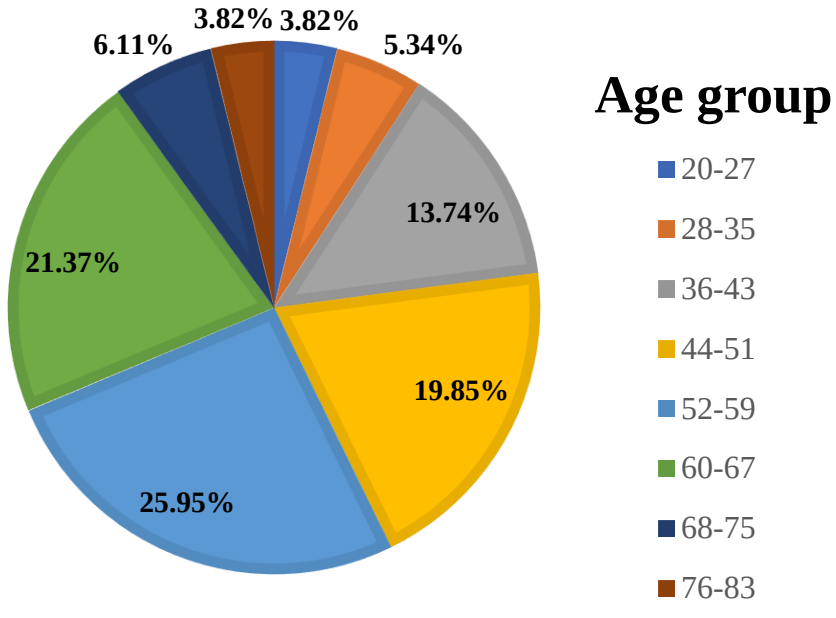

Figure 13: Age distribution in CLD patients in this study.

5.1.4 Viremia in IDU population:

Overall, 65 anti-HCV-positive blood samples were collected from drug users (PWIDs) with the help of our collaborative NGOs. Surprisingly, all of them were male and it was reconfirmed during this study that no female was found. Out of 65 anti-HCV positive samples 46 were found to be RNA positive. Demographic and other socio-economic data were scooped out from the PWIDs population. But it was not that easy. Except for age other data were very scarce due to the conserved behaviour of PWIDs population groups (**Table 9**).

N=65 Variables		RNA (+) n= 46	RNA (-) n=19	Viremia (%) 70.70%	p-value
Gender	Male	46	19	70.70%	-
	Female	-	-		
Age-group	14-24	2	2	50.00	0.60
	25-35	18	7	72.00	
	36-46	19	5	79.17	
	47-57	4	3	57.14	
	>58	3	2	60.00	

Table 9: HCV viremia in PWIDs population in this study.

Other groups Haemophilia and HIV with HCV groups did not contain enough samples for proper statistical analysis. Therefore, detailed results were avoided for these groups.

5.1.5 HCV modulated disease complications (Pathogenesis) in high-risk group population:

5.1.5.1 HCV pathogenesis in CKD patients:

Primary cause of ESRD, (N=130)	HCV (+) (n=99)	HCV (-) (n= 31)	p-value
Glomerulonephritis, vasculitis	9	2	**0.04***
Cystic/hereditary/congenital	10	6	
Diabetes	14	8	
Obstruction	17	10	
Interstitial nephritis	20	2	
Hypertension	23	2	
Other cause of ESRD	6	1	

Table 10: HCV infection associated with ESRD in CKD patients in this study.

****Statistically significant.***

ESRD, or End Stage Renal Disease often augmented by HCV in CKD patients, causes serious complications, and increases morbidity in CKD patients. Out of 155 patients, 130 patients had a history of several types of ESRD. The table **(Table 10)** clearly shows that HCV infection correlated with ESRD.HCV also causes an increased risk of hepatorenal complications. Out of 155 CKD patients, 25 patients had a history of liver cirrhosis. Out of these 25 patients, 20 patients were found as HCV RNA-positive (**Table 11**).

Total = 155	HCV (+)	HCV (-)	p-value
Cirrhosis	20	5	**0.001***
No-cirrhosis	58	72	

Table 11: HCV infection associated with Cirrhosis in CKD patients in this study.

5.1.5.2 HCV pathogenesis in β-thalassemia:

HCV also increases liver complications in thalassemia patients. HCV-RNA-positive patients show higher levels of liver enzyme profiles than HCV-negative (**Table 12**).

Biochemical parameters	HCV (+)	HCV (-)	p-value
Haemoglobin (g/dL)	7.4 ± 1.45	7.7 ± 1.11	0.353
Total leukocyte count (/microL)	6069.6 ± 2121.33	5840.4 ± 1809.12	0.573
Platelet count (X 10^9 /L)	130 (100 – 160)	130 (100 – 180)	0.06
Urea (mg/dL)	25.9 ± 8.21	23.7 ± 4.54	0.075
Creatinine (mg/dL)	0.52 ± 0.158	0.52 ± 0.159	0.912
Total cholesterol (mg/dL)	105.7 ± 23.53	105.0 ± 18.35	0.895
Triglycerides (mg/dL)	**147.9 ± 39.70**	**131.2 ± 23.77**	**0.007***
Bilirubin (mg/dL)	**2.4 (1.5 – 3.9)**	**2.1 (1.6 – 2.6)**	**0.014***
Alanine aminotransferase (IU/L)	**121.4 ± 91.95**	**60.9 ± 35.29**	**0.004***
Aspartate aminotransferase (IU/L)	**105.8 ± 75.51**	**56.9 ± 42.01**	**< 0.001***
Alkaline phosphatase (U/L)	**157.0 (128.0 – 180.0)**	**124.0 (112.0 – 149.0)**	**0.046***
Albumin (g/dL)	4.1 ± 0.43	3.9 ± 0.46	0.115
Globulin (g/dL)	3.3 ± 0.59	3.3 ± 0.63	0.314
Prothrombin Time	15.7 ± 2.21	14.9 ± 0.94	0.099

Table 12: HCV infection associated with pathogenesis in β- thalassemia patients in this study.

****Statistically significant.***

5.2 Genomic characterization of the isolated HCV strains in different high-risk group populations:

5.2.1 Genotype Distribution and phylogenetic analysis of HCV isolates from the thalassemia population:

5.2.1.1 Genotype distribution of HCV isolates from the thalassemia population:

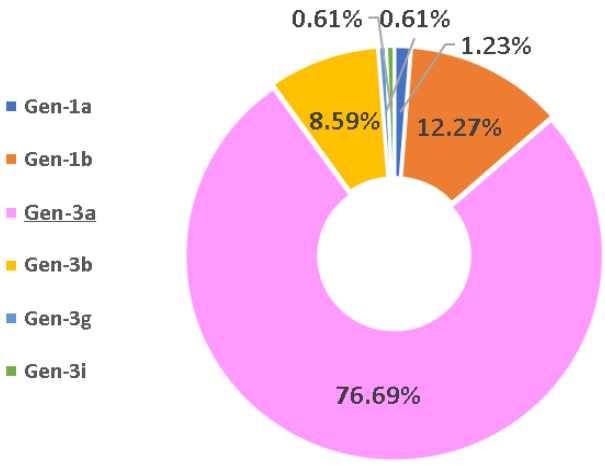

Figure 14: HCV genotype distribution in β-Thalassemia patients in this study.

Out of 163 HCV RNA-positive samples, isolated from the thalassemia population, 125 isolates (76.69%) belonged to HCV genotype-3a, which is the most prevalent genotype in the thalassemia population, followed by Genotype 1b (12.26%, n=20) and genotype 3b (8.58%, n=14). Rarely reported Genotype 3g and Genotype 3i were also found in very small percentages (0.6%, n=1) (**Figure 14**).

5.2.1.2 Phylogenetic analysis of HCV isolates found from the thalassemia population:

Phylogenetic analysis was performed using core sequences isolated from HCV-infected thalassemia patients. The first 300bp sequence (371- 671nt of the H77-accession number NC-004102) of the core region was used for the analysis. Phylogenetic analysis was performed using 120 representative partial core sequences from HCV RNA-positive samples and 102

reference sequences from NCBI. To investigate the evolutionary linkage among lab strains and reference strains, partial core sequences of Eighty-six laboratory-isolated 3a strains (**Accession Numbers PP495548-PP495633**), Twenty-one 1b strains (**Accession Numbers, PP495635-PP495655**), Ten 3b strains **(Accession Numbers, PP495656-PP495665**), one 3g strain (**Accession number-PP495666**), one 3i (**Accession number-PP495668**) and one 1a strain (**Accession number- PP495669**) were aligned with HCV reference strains using Molecular Evolutionary Genetics Analysis tool (MEGA-X). The evolutionary history was inferred by using the Maximum Likelihood method and the Tamura-Nei models. There were 222 nucleotide sequences in this analysis. Less than 5% of alignment gaps, missing data, and

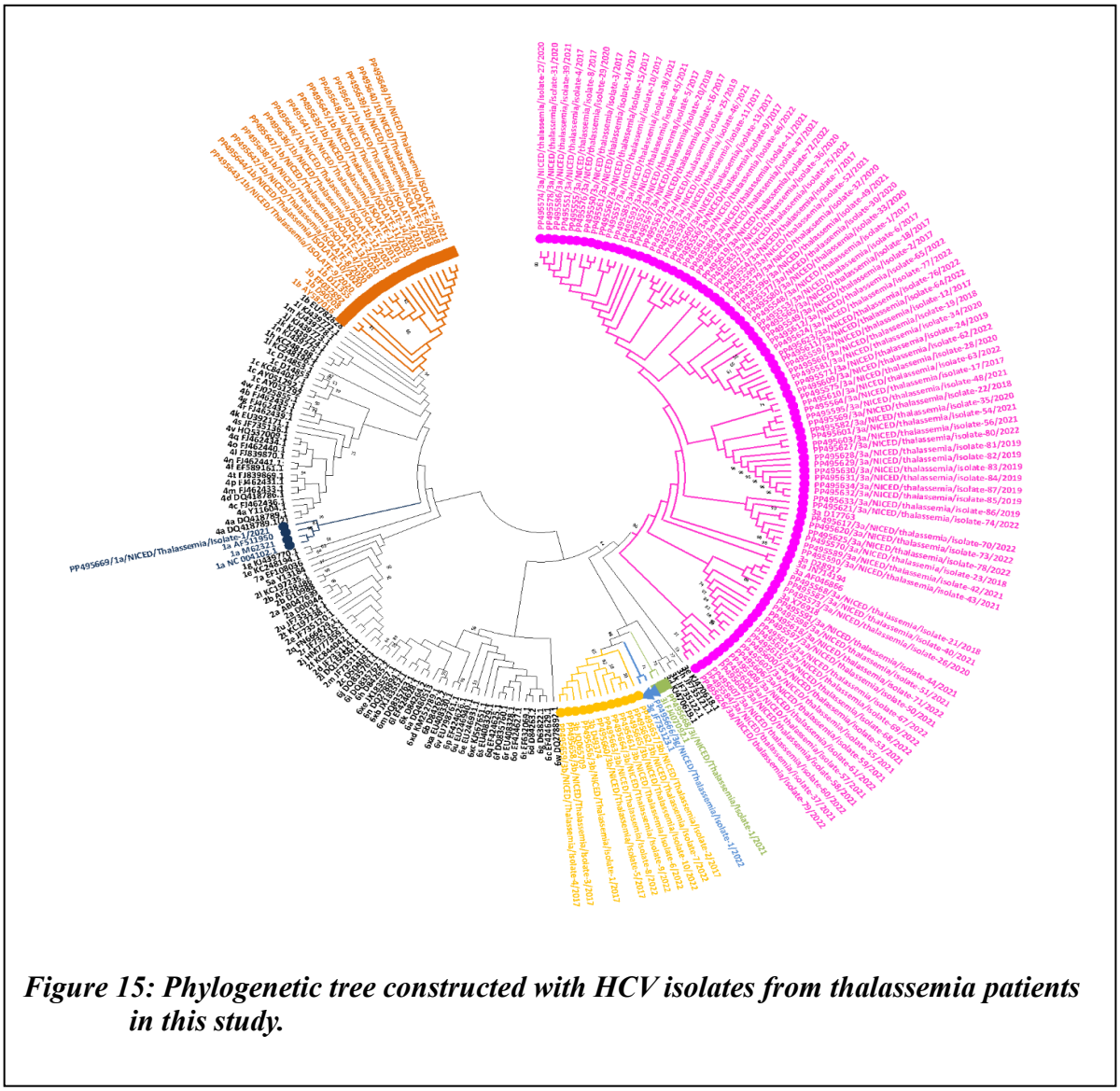

Figure 15: Phylogenetic tree constructed with HCV isolates from thalassemia patients in this study.

unambiguous bases were permitted at any position (partial deletion option), and all positions with less than 95% site coverage were deleted. The final dataset contained 300 locations in total (**Figure 15**).

5.2.2 Genotype Distribution and phylogenetic analysis of HCV isolates from the CKD population:

5.2.2.1 Genotype distribution of HCV isolates from the CKD population:

Out of 78 HCV RNA-positive samples from the CKD population, 53 samples (67.95%) were found to be genotype 1c, 8 samples (10.26%) were found to be genotype 1a, 6 samples (7.69%) were found to be genotype 4a, 5 samples (6.40%) were found to be genotype 3a, 2 samples (2.56%) were found to be genotype 1b and 2 samples (2.56%) were found to be genotype 3b (**Figure 16**).

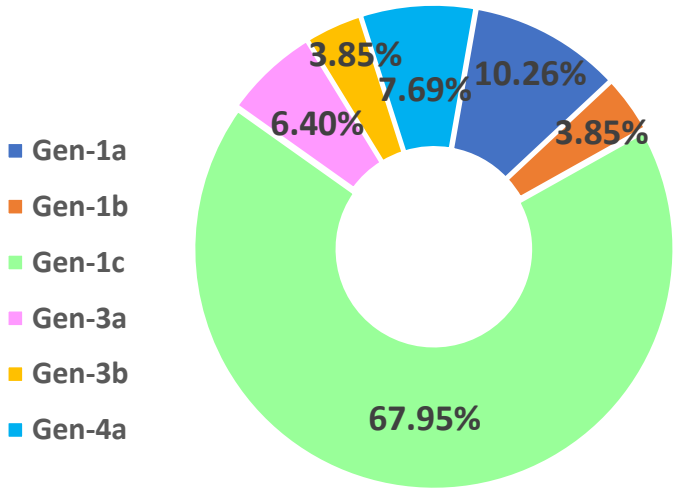

Figure 16: HCV genotype distribution in CKD patients in this study.

5.2.2.2 Phylogenetic analysis of HCV isolates found from CKD population:

Core sequences derived from CKD patients infected with HCV were used for phylogenetic analysis. For the analysis, the first 300bp sequence of the core region (respective 371–671nt of the H77–accession number NC–004102) was utilized. A phylogenetic tree was constructed

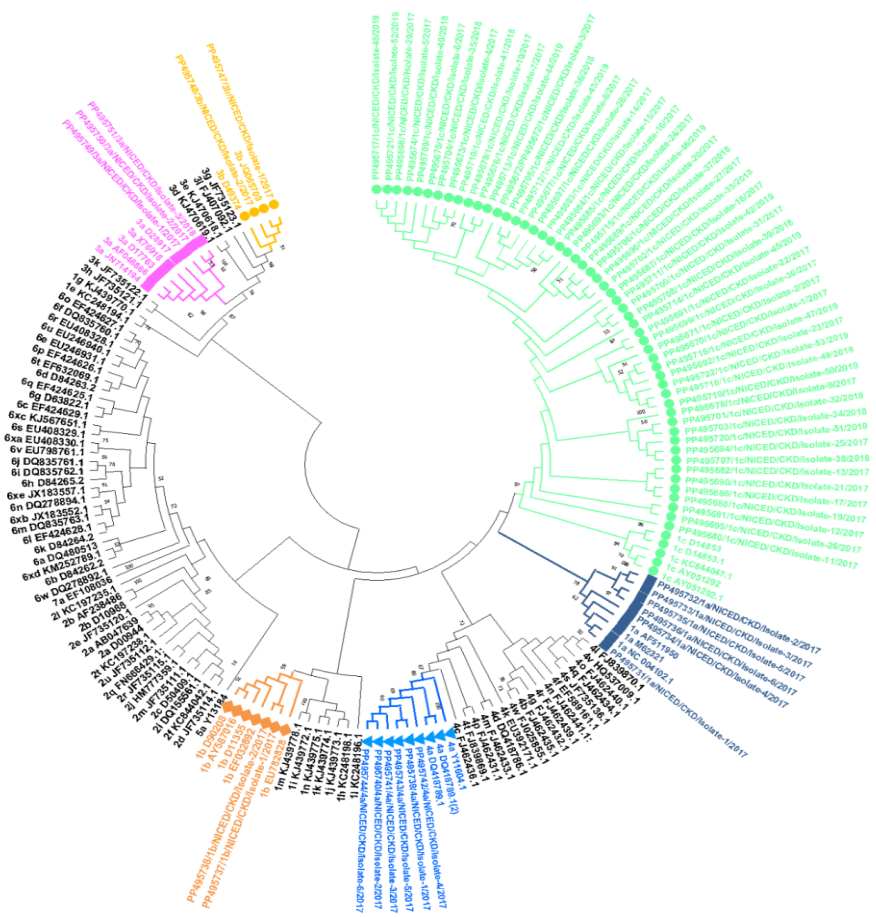

Figure 17: Phylogenetic tree constructed with HCV isolates from CKD patients in this study.

by MEGA-X software with 71 representative partial core gene sequences (300 bp) which were also submitted to the Gen Bank [1c (n=52, **PP495670-PP495721**), 1a (n=6, **PP495731-PP495736**),1b (n=2, **PP495737-PP495738**), 4a (n=6, **PP495739- PP495744**), 3b (n=2, **PP495747- PP495748**), 3a (n=3, **PP495749- PP495751**)] along with 102 reference sequences

downloaded from NCBI. All these sequences were aligned with the inbuilt MUSCLE program of MEGA-X. The evolutionary history was inferred by using the maximum likelihood method and the Tamura-Nei models. There were 88 nucleotide sequences in this analysis. Less than 5% of alignment gaps, missing data, and unambiguous bases were permitted at any position (partial deletion option), and all positions with less than 95% site coverage were deleted. The final dataset contained 300 locations in total (**Figure 17**).

5.2.3 Genotype Distribution and phylogenetic analysis of HCV isolates from the CLD population:

5.2.3.1 Genotype distribution of HCV isolates from the CLD population:

Within 74 HCV RNA-positive CLD samples, the most prevalent HCV genotype was genotype 3a (36.71%, n=29), followed by genotype 3b (34.18%, n=27) and genotype 1b (16.46%, n=8). Other genotypes such as genotype 1a, 1c, 3g and 3i were also found with prevalence rates of 5.06% (n=4), 2.53% (n=2), 3.80% (n=3) and 1.27% (n=1) respectively (**Figure 18**).

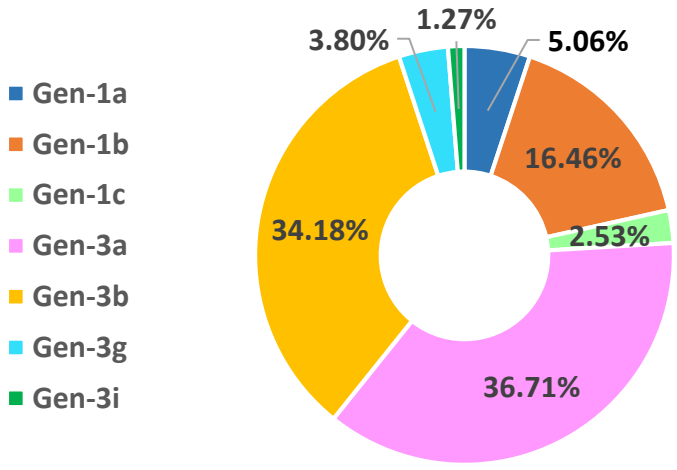

Figure 18: HCV genotype distribution in CLD patients in this study.

5.2.3.2 Phylogenetic analysis of HCV isolates from the CLD population:

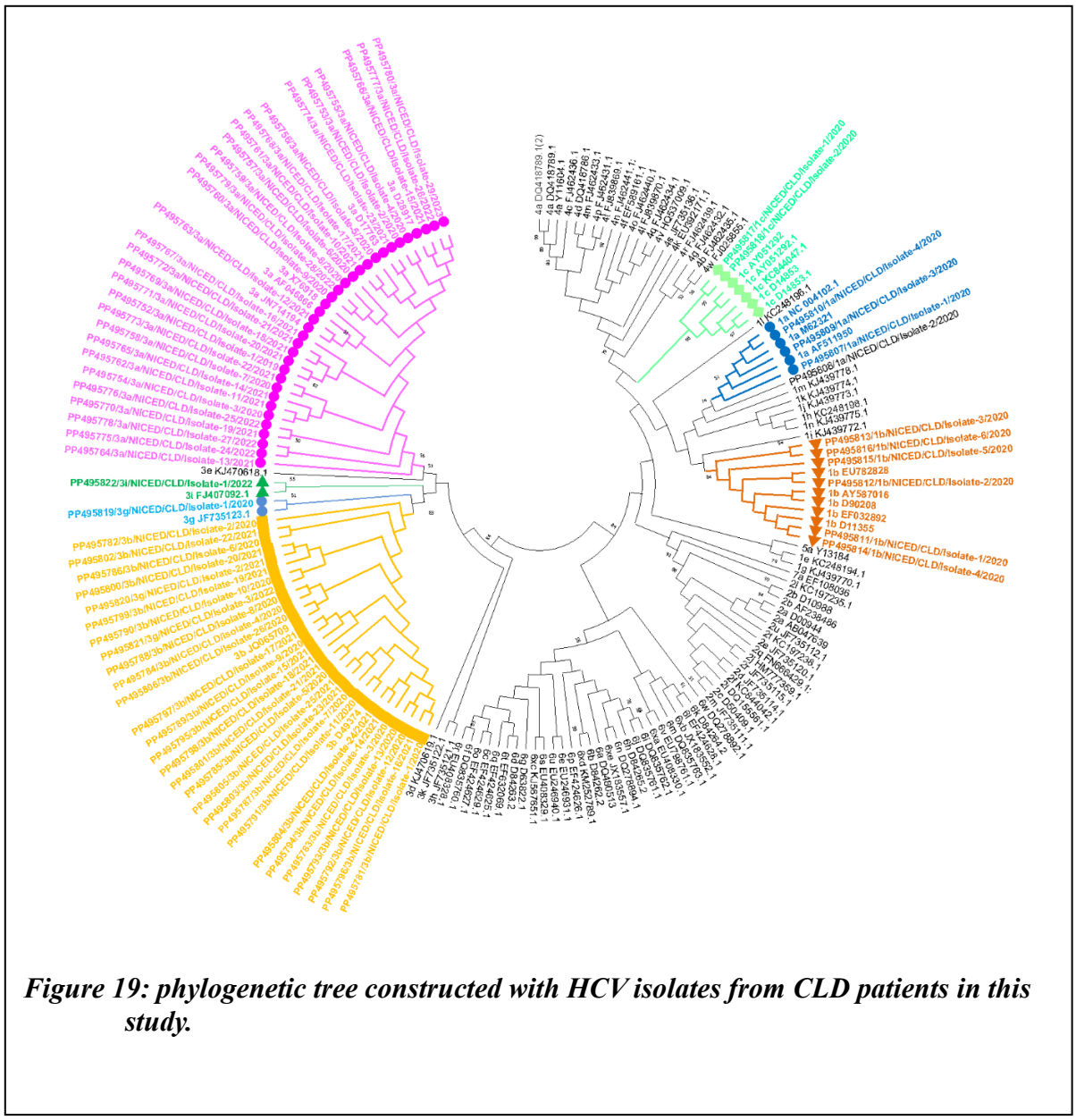

Figure 19: phylogenetic tree constructed with HCV isolates from CLD patients in this study.

To investigate the evolutionary relationships among the HCV sequences derived from the HCV-infected CLD patients, a representative phylogenetic tree was built using the Molecular Evolutionary Genetics Analysis software (MEGA version X). The first 300bp of the partial-core gene sequence, or 371nt to 671nt of the H77-accession number NC-004102, were aligned from the 405bp of the partial-core gene sequence, using the CLUSTAL OMEGA program in MEGA-X and 41 reference sequences of different HCV genotypes and subtypes that were retrieved from NCBI. Using the Kimura 2-parameter model as the best-fit model, the maximum

likelihood method was applied to generate the evolutionary tree. HCV sequences of core gene were submitted to NCBI under the following accession numbers- **PP495752-PP495780** (Gen-3a), **PP495781-PP495806** (Gen-3b), **PP495807-PP495810** (Gen-1a), **PP495811- PP495816** (Gen-1b) and **PP495817- PP495818** (Gen-1c), **PP495819 - PP495821** (Gen-3g) and **PP495822** (Gen-3i) and used for this analysis. The tree with the highest log likelihood (-3780.04) is shown. This analysis involved 108 nucleotide sequences. There was a total of 300 positions in the final dataset (**Figure 19**).

A strange paradox was found in this study about HCV genotype distribution. In thalassemia patients most prevalent genotype was genotype -3a (76.69%). Whereas in the CKD patient population, the most common genotype was genotype-1c (67.95%) but in the case of CLD patients genotype-3a (36.71%) and 3b (34.18%) were almost equally distributed (**Figure 20**).

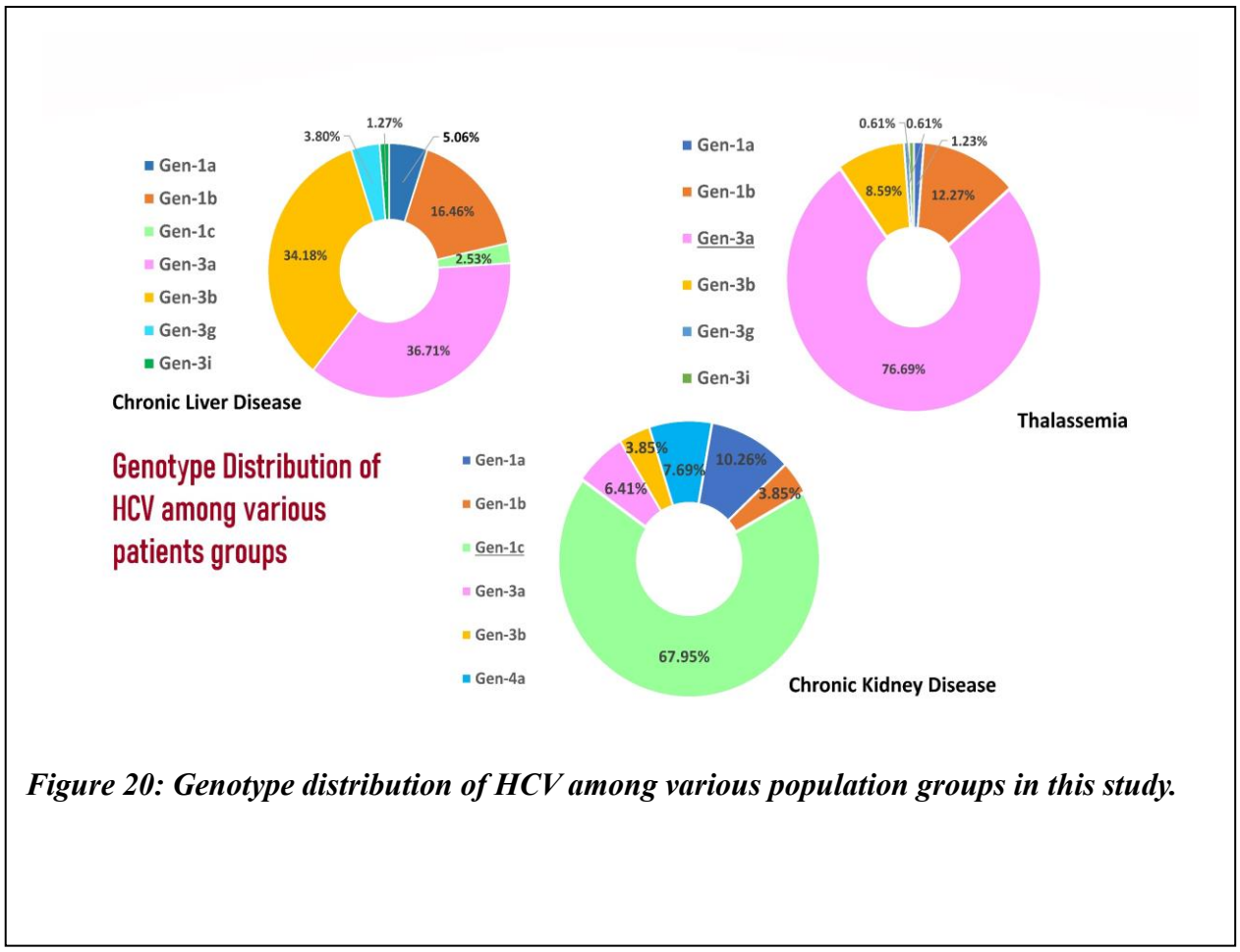

Figure 20: Genotype distribution of HCV among various population groups in this study.

5.2.4 Phylogeographical relations of prevalent HCV subtypes isolated from West Bengal:

5.2.4.1 Distribution of HCV subtype 3a:

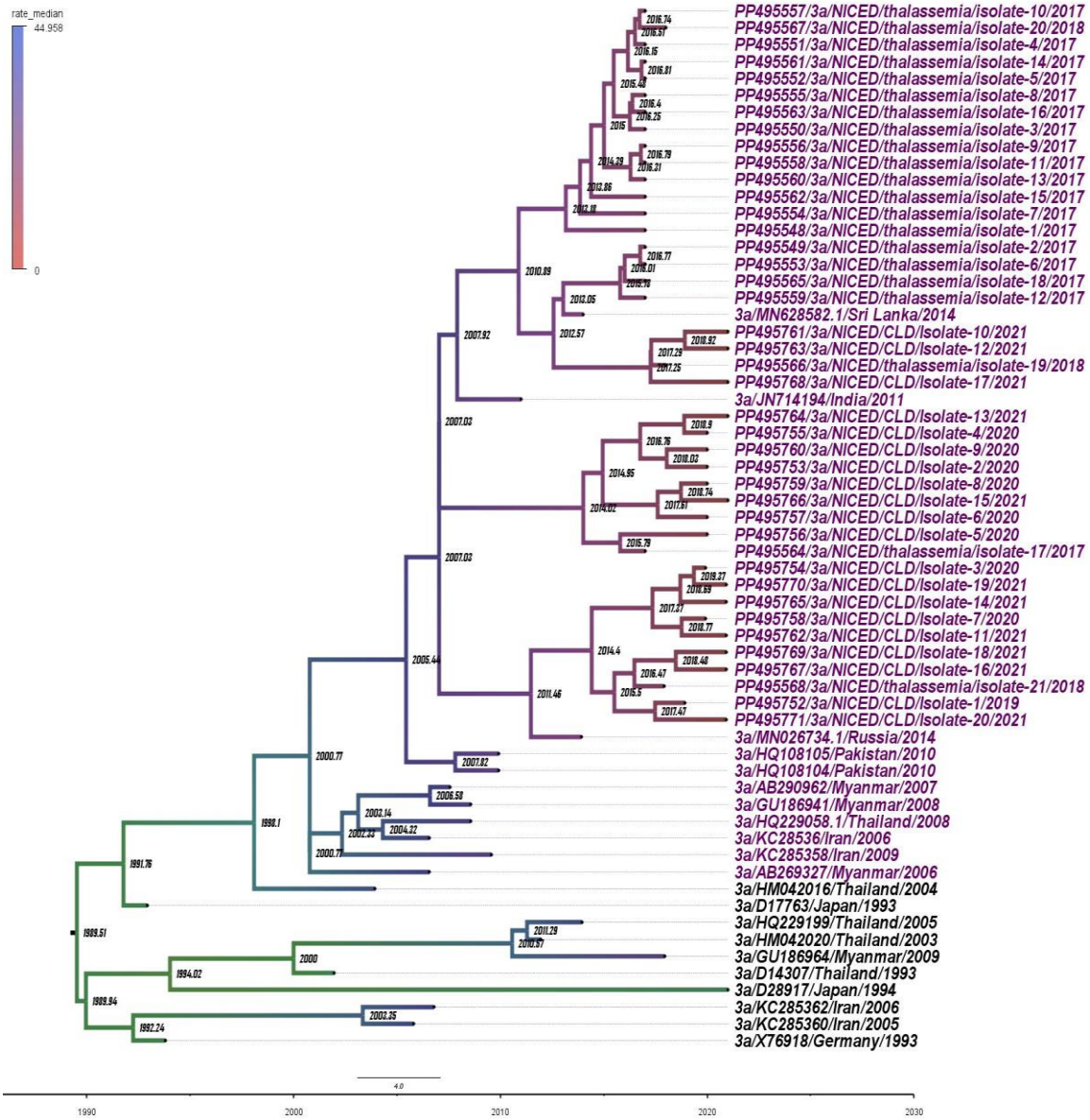

Figure 21: Phylogeographical distribution of HCV 3a isolates found in this study with other countries' isolates.

HCV subtype 3a was found to be the most prevalent HCV subtype in this region. Phylogeographical analysis revealed that the study isolates showed relatedness with Indian subcontinental and Southeast Asian isolates. Study isolates were found to share their common

ancestors with countries like Sri Lanka, Russia, Pakistan, Myanmar, Iran, and Thailand (**Figure 21**).

5.2.4.2 Distribution of HCV subtype 1c:

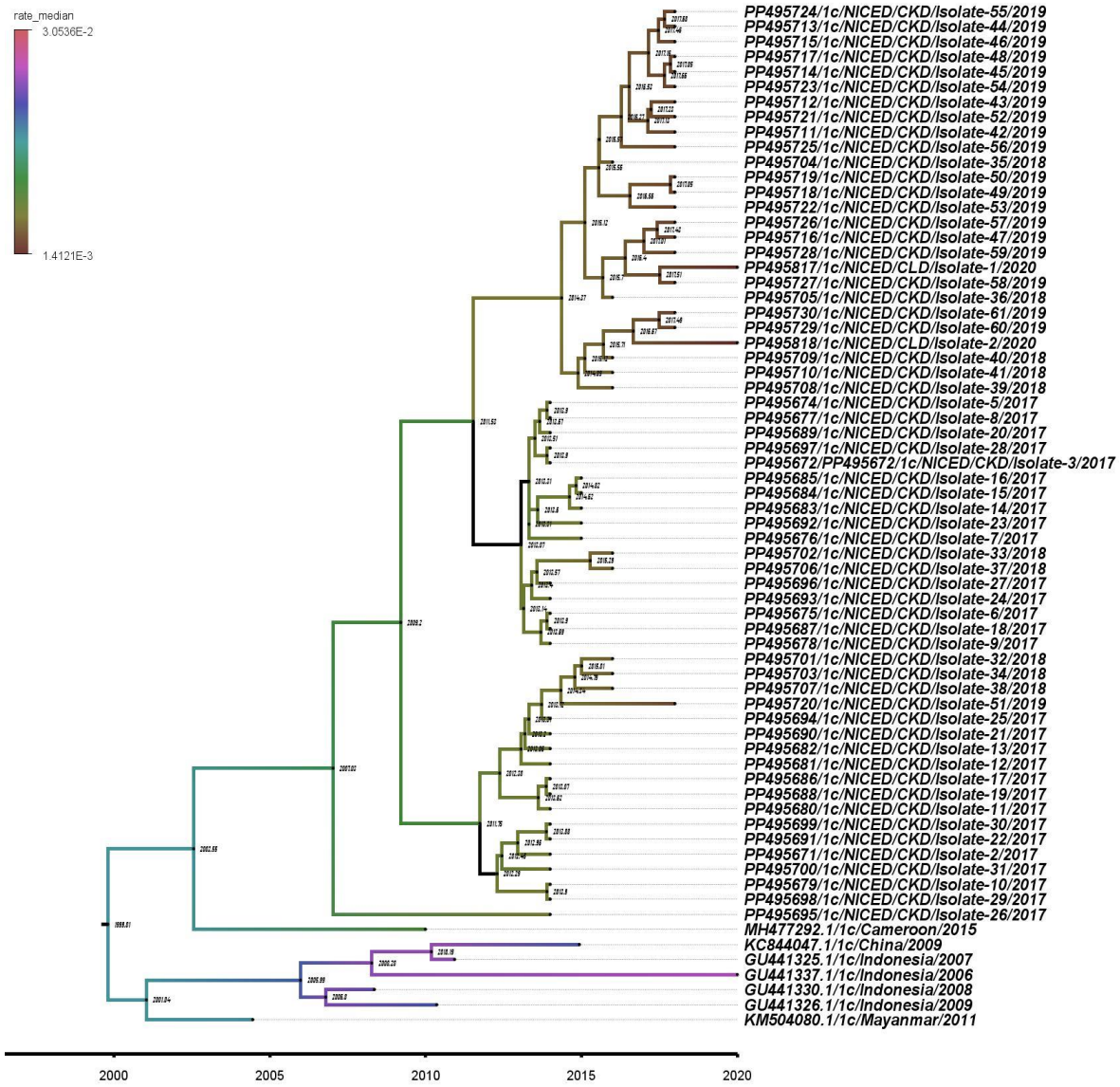

Figure 22: Phylogeographical distribution of HCV 1c isolates found in this study with other countries' isolates.

HCV subtype 1c was found to be the second most prevalent subtype in this region although subtype 1c prevalence was restricted to only the CKD population group. Other high-risk groups or the general population with CLD had been rarely infected with 1c isolates. Subtype 1c is a

very rare subtype which can be mostly found in Indonesia or some African countries. Study isolates showed their relations with Indonesia, China, Cameroon, and Myanmar (**Figure 22**).

5.2.4.3 Distribution of HCV subtype 3b:

The 3rd most common HCV subtype found in this region was 3b. Study isolates were found to share common ancestors with China, Myanmar, Vietnam, Thailand, and other Southeast Asian isolates (**Figure 23**).

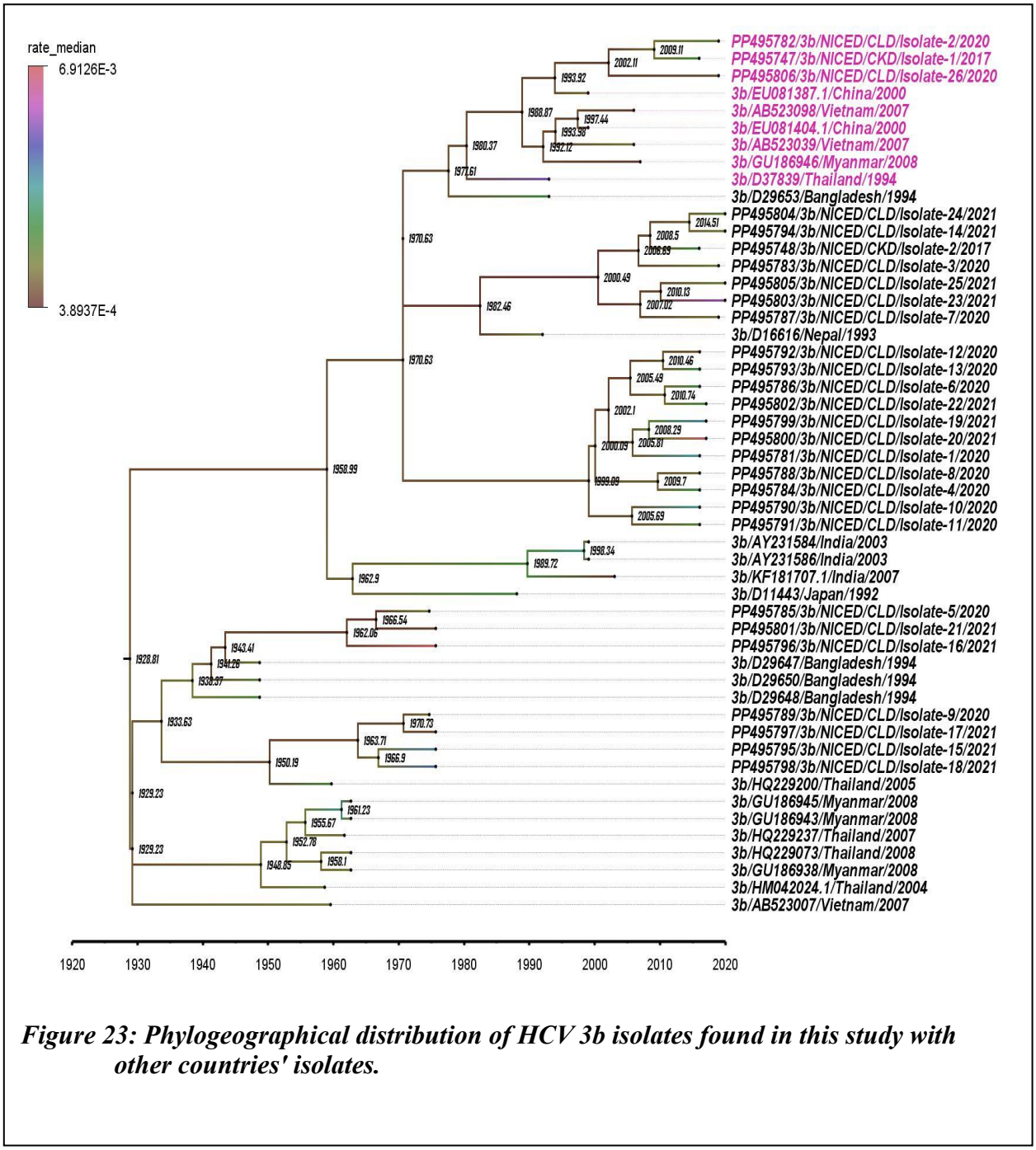

Figure 23: Phylogeographical distribution of HCV 3b isolates found in this study with other countries' isolates.

5.2.4.4 Distribution of HCV subtype 1b:

The 4th most prevalent HCV subtype was 1b. This study found that HCV isolates in this study showed their relatedness with countries like Japan, Myanmar, and Thailand (**Figure 24**).

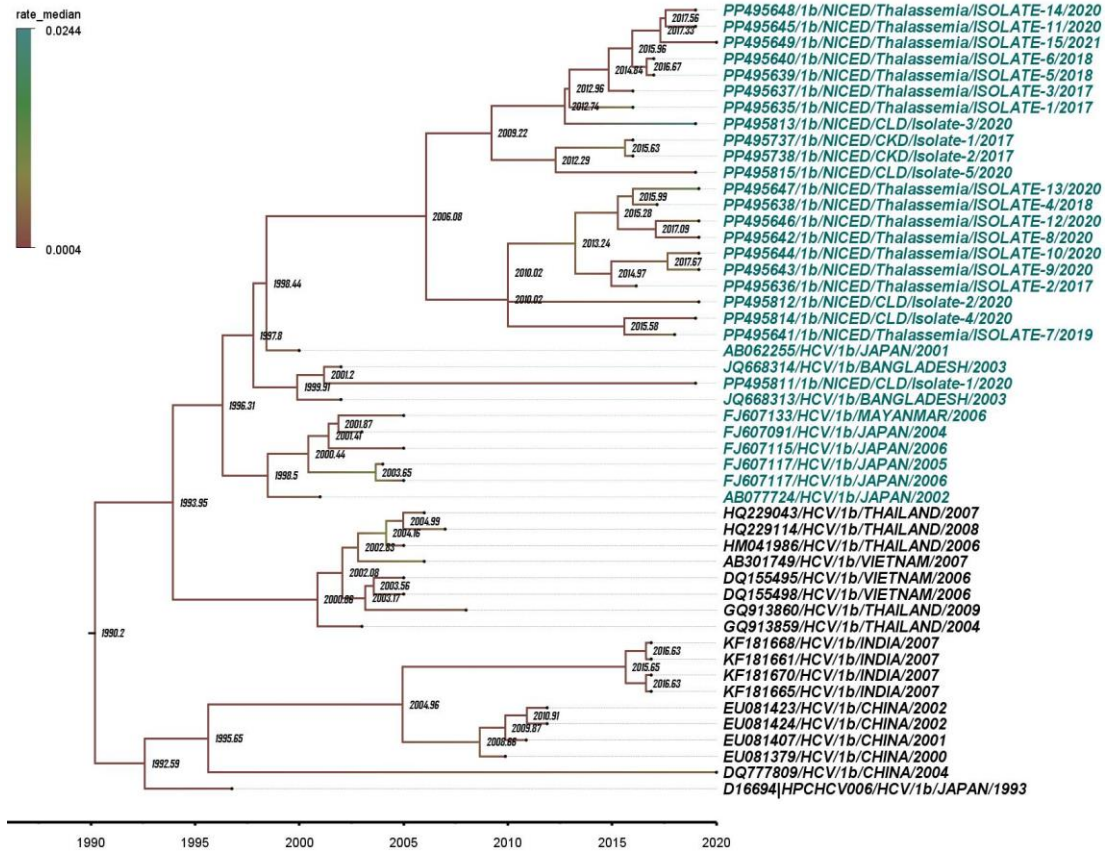

Figure 24: Phylogeographical distribution of HCV 1b isolates found in this study with other countries' isolates.

5.2.4.5 Distribution of HCV subtype 1a:

HCV subtype 1a is found worldwide. In this study, HCV subtype 1a is also found. HCV isolates in this study share relatedness with various countries' isolates like China, Venezuela, Germany, and Iran (**Figure 25**).

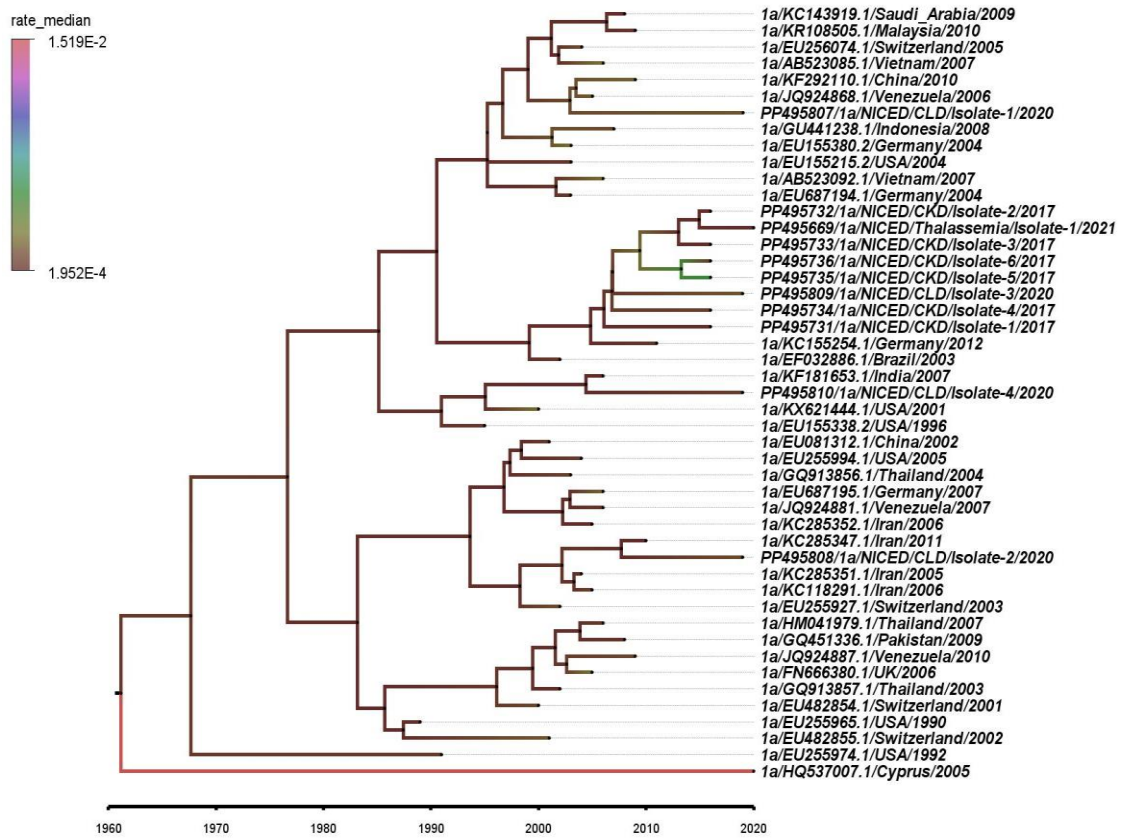

Figure 25: Phylogeographical distribution of HCV 1a isolates from West Bengal with other countries' isolates.

5.2.4.6 Distribution of HCV genotype 4a:

HCV subtype 4a was found with a significant prevalence (~7%) among the CKD population in this region. However, it is also a rare subtype for the Indian scenario and can be prevalently found in Egypt and the Middle East. Study isolates also share similarities with Saudi-Arabian isolates (**Figure 26**).

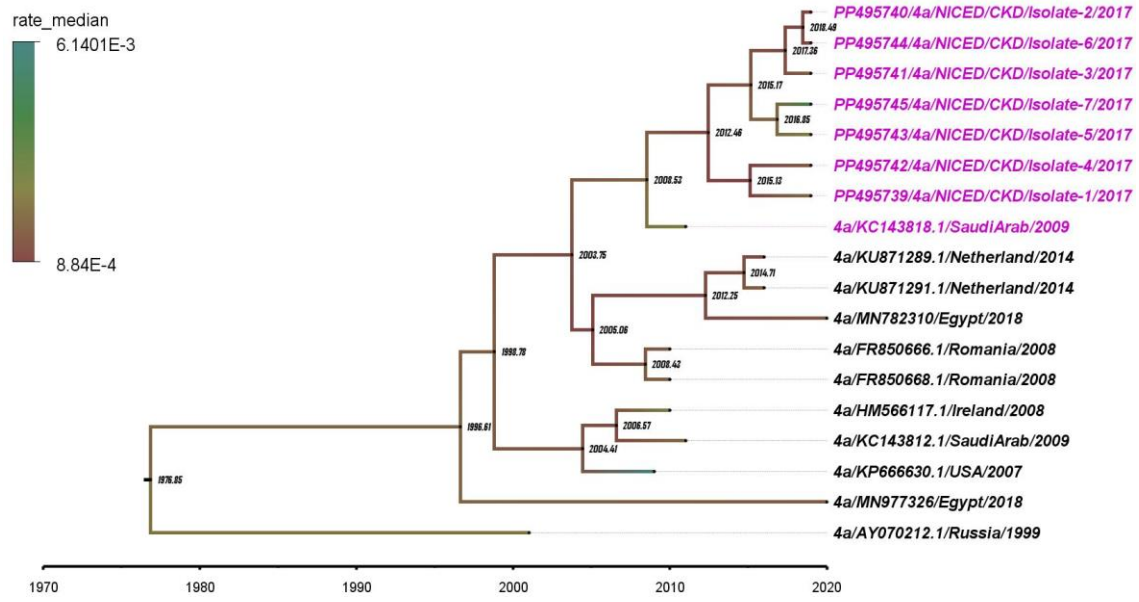

Figure 26: Phylogeographical distribution of HCV 4a isolates from West Bengal with other countries' isolates.

5.3 Mutational analysis of HCV non-structural gene 3 (NS3) and to find out the important mutations which are associated with chronic HCV and prediction of treatment outcome:

5.3.1 Amplification of HCV NS3 gene and sequencing:

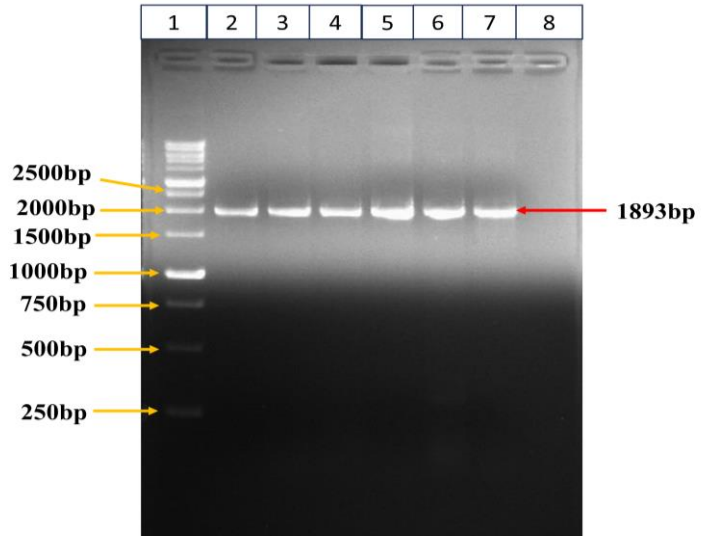

Figure 27: Amplification of HCV NS3 gene by PCR. Lane 1: DNA ladder, Lane 2-7: position of NS3 in agarose gel, Lane 8: Negative control.

HCV NS3 gene (1893bp) was amplified by the nested PCR and analysed in 1% agarose gel. 1893 bp long band confirmed NS3 region (**Figure 27**). 1893bp PCR amplified band was cut

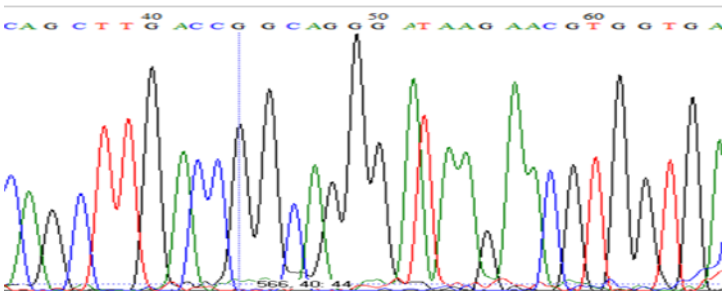

Figure 28: Electropherogram of NS3 Sequencing.

and purified with a Gel and PCR cleanup kit. Purified PCR products were sequenced by Sanger sequencing. The electropherogram found by Sanger sequencing **(Figure** 28) was analysed with the Bio-Edit tool. All sequences were also reported to NCBI for accession numbers (**Accession numbers: OP616746-OP616781**).

5.3.2 Translation of NS3 sequences into protein sequences and mutational analysis:

NS3-specific nucleotide sequences of all 36 genotype 3a CLD samples were converted to amino acid sequences by the Expasy server. Amino acid sequences were aligned with the CLUSTAL omega program built-in MEGA software. To find important mutations aligned sequences were subjected to Bio-Aider software and 'HCV GLUE' server.

5.3.2.1 A possible NS3 mutation which may lead to decompensated liver Cirrhosis:

Out of all 36 patients with chronic liver disease, 10 patients were at the stage of Decompensated Chronic Liver disease (DCLD), and the rest 26 were at the stage of Compensated stage. Bio-Aider software analysis found that the '**N224T'** mutation was present at a higher rate in (DCLD) samples compared to the other 26 samples. 'N224T' mutation was found in 70% of

Total sample collected = 36
Chronic liver disease (CLD) = 26
Decompensated liver disease (DCLD) =10
N224T mutation present in CLD = 8/26 = 30.76%
N224T mutation present in DCLD = 7/10 = 70%

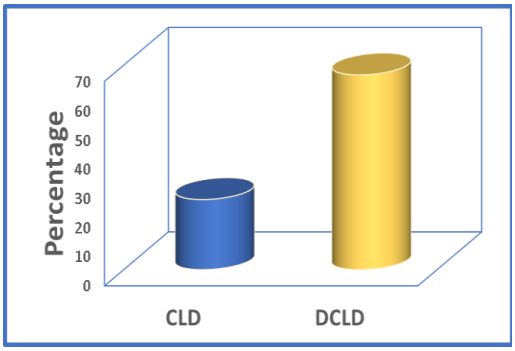

Figure 29: Percentage of N224T mutation found in CLD and DCLD patients.

cases in the case of DCLD patients, whereas 'N224T' mutation was present at a lower rate (30.76%) in CLD samples (**Figure 29**).

5.3.2.2 Important mutations found by HCV-GLUE server:

HCV-GLUE is a bioinformatics resource for HCV sequence data (http://hcv-glue.cvr.gla.ac.uk/#/home, accessed on 19/06/2021). This resource is very important for drug

resistance studies or treatment planning. Generally, Drug resistance findings are assigned to one of three categories according to the strength of evidence for drug resistance. Those are-

Category I polymorphisms have the strongest evidence: either (a) in vitro resistance level ≥ 5 and found at baseline or treatment-emergent in vivo, or (b) both found at baseline and treatment-emergent.

Category II: in vitro level ≥ 50 or found at baseline or treatment-emergent.

Category III: in vitro level ≥ 5

In this study, naturally occurring Category II mutations were observed at a very high rate against NS3 inhibitors like Glecaprevir, Paritaprevir and Grazoprevir. Only, the '**Y56Y+Q168Q+I/V170I'** mutation was found in 75% of cases, and the '**Y56Y+Q168Q+I/V170I'** mutation along with '**A166S'** was found in 22% of cases and only 3% of cases did not have any drug-resistant mutations in them (**Figure 30**).

Mutation	Category	Resistance towards antiviral
Y56Y+Q168Q+I/V170I	II	**Grazoprevir**
A166S, Y56Y+Q168Q+I/V170I	II	**Glecaprevir, Paritaprevir, Grazoprevir**

Table 13: Naturally occurring drug-resistant NS3 mutations were found in this study.

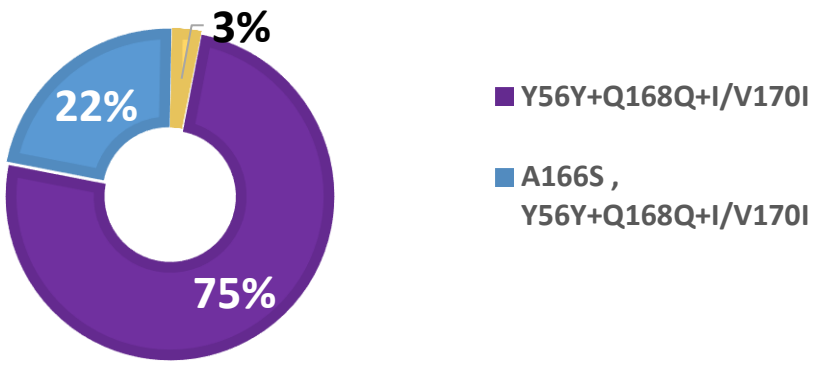

Figure 30: Percentage of NS3 drug-resistant mutations present among patients.

5.4 Prediction of conserved NS3-specific T-cell epitopes:

All 36 NS3 amino acid sequences were aligned and a consensus sequence was generated. This consensus sequence was subjected to the IEDB web server for epitope prediction.

5.4.1 Prediction of MHC-I and MHC-II Epitopes:

A total of 119 MHC-I epitopes were predicted and 436 MHC-II epitopes were predicted by the IEDB web server. Only epitopes which had a percentile rank >0.50 were taken for analysis.

MHC-I Binding Prediction Results

Method used: netmhcpan_el

allele	seq_num	start	end	length	peptide	score	percentile_rank
HLA-B*35:01	1	259	267	9	VPTGASLTY	0.993065	0.01
HLA-B*58:01	1	570	578	9	RAQASPPSW	0.990937	0.01
HLA-A*68:02	1	311	319	9	ETAGVRLTV	0.986888	0.01
HLA-B*57:01	1	570	578	9	RAQASPPSW	0.986857	0.01
HLA-A*01:01	1	592	600	9	LTGPTPLLY	0.97955	0.01
HLA-A*68:01	1	122	130	9	STASLLSPR	0.969748	0.01
HLA-B*35:01	1	189	197	9	TPPAVPQSY	0.961558	0.02
HLA-A*11:01	1	352	360	9	KAIPIALLK	0.957145	0.01
HLA-A*68:01	1	16	24	9	EIVTSLTGR	0.949498	0.03
HLA-B*07:02	1	85	94	10	WPAPPGAKSL	0.943482	0.04
HLA-B*44:03	1	507	516	10	AETTVRLRAY	0.943017	0.03
HLA-A*03:01	1	352	360	9	KAIPIALLK	0.936696	0.02
HLA-A*02:01	1	13	21	9	LLGEIVTSL	0.931414	0.03
HLA-A*02:03	1	559	567	9	YLAAYQATV	0.930926	0.02
HLA-B*15:01	1	551	559	9	KQQGLNFSY	0.930103	0.01
HLA-B*35:01	1	193	201	9	VPQSYQVGY	0.926895	0.03
HLA-A*02:01	1	559	567	9	YLAAYQATV	0.924399	0.03
HLA-A*68:01	1	54	62	9	TVYHGAGSR	0.919006	0.06
HLA-B*44:02	1	507	516	10	AETTVRLRAY	0.91319	0.02
HLA-B*57:01	1	45	53	9	GTTVGGVMW	0.913	0.09
HLA-B*07:02	1	505	513	9	QPAETTVRL	0.91133	0.04
HLA-B*15:01	1	35	43	9	VLSTATQTF	0.908837	0.02
HLA-A*01:01	1	342	350	9	GSEGEIPFY	0.907844	0.03
HLA-B*07:02	1	589	598	10	KPTLTGPTPL	0.9056	0.04
HLA-B*58:01	1	592	600	9	LTGPTPLLY	0.895804	0.06
HLA-A*30:02	1	551	559	9	KQQGLNFSY	0.893065	0.01
HLA-A*01:01	1	591	600	10	TLTGPTPLLY	0.881809	0.04
HLA-B*58:01	1	45	53	9	GTTVGGVMW	0.880525	0.07
HLA-A*68:01	1	121	130	10	DSTASLLSPR	0.879576	0.1
HLA-B*35:01	1	258	267	10	TVPTGASLTY	0.876082	0.05
HLA-B*40:01	1	432	440	9	VEQYVDFSL	0.875826	0.07
HLA-B*57:01	1	44	53	10	LGTTVGGVMW	0.875091	0.14
HLA-B*57:01	1	246	255	10	YGIEPNIRNW	0.874708	0.14
HLA-A*02:03	1	13	21	9	LLGEIVTSL	0.869595	0.04
HLA-A*68:01	1	3	11	9	ITAYAQQTR	0.868095	0.12
HLA-B*53:01	1	259	267	9	VPTGASLTY	0.86405	0.03
HLA-A*30:02	1	210	218	9	KSTNVPAAY	0.859627	0.01

Figure 31: MHC-I epitope prediction (a snapshot).

MHC-II Binding Prediction Results

Method used: recommended

allele	seq_num	start	end	length	method	peptide	percentile_rank	adjusted_rank	comblib_core	comblib_score	comblib_rank	comblib_adjusted_rank	smm_align_core	smm_align_ic50
HLA-DRB3*02:02	1	222	236	15	NetMHCIIpan	GYNVLVLNPSVAATL	0.01	0.01	-	-	-	-	-	-
HLA-DRB3*02:02	2	222	236	15	NetMHCIIpan	GYNVLVLNPSVAATL	0.01	0.01	-	-	-	-	-	-
HLA-DRB3*02:02	1	224	238	15	NetMHCIIpan	NVLVLNPSVAATLGF	0.01	0.01	-	-	-	-	-	-
HLA-DRB3*02:02	2	224	238	15	NetMHCIIpan	NVLVLNPSVAATLGF	0.01	0.01	-	-	-	-	-	-
HLA-DRB3*02:02	1	221	235	15	NetMHCIIpan	QGYNVLVLNPSVAAT	0.01	0.01	-	-	-	-	-	-
HLA-DRB3*02:02	2	221	235	15	NetMHCIIpan	QGYNVLVLNPSVAAT	0.01	0.01	-	-	-	-	-	-
HLA-DRB3*02:02	1	221	236	16	NetMHCIIpan	QGYNVLVLNPSVAATL	0.01	0.01	-	-	-	-	-	-
HLA-DRB3*02:02	2	221	236	16	NetMHCIIpan	QGYNVLVLNPSVAATL	0.01	0.01	-	-	-	-	-	-
HLA-DRB3*02:02	1	223	237	15	NetMHCIIpan	YNVLVLNPSVAATLG	0.01	0.01	-	-	-	-	-	-
HLA-DRB3*02:02	2	223	237	15	NetMHCIIpan	YNVLVLNPSVAATLG	0.01	0.01	-	-	-	-	-	-
HLA-DRB3*02:02	1	220	235	16	NetMHCIIpan	AQGYNVLVLNPSVAAT	0.02	0.02	-	-	-	-	-	-
HLA-DRB3*02:02	2	220	235	16	NetMHCIIpan	AQGYNVLVLNPSVAAT	0.02	0.02	-	-	-	-	-	-
HLA-DRB3*02:02	1	222	237	16	NetMHCIIpan	GYNVLVLNPSVAATLG	0.02	0.02	-	-	-	-	-	-
HLA-DRB3*02:02	2	222	237	16	NetMHCIIpan	GYNVLVLNPSVAATLG	0.02	0.02	-	-	-	-	-	-
HLA-DRB3*02:02	1	223	238	16	NetMHCIIpan	YNVLVLNPSVAATLGF	0.02	0.02	-	-	-	-	-	-
HLA-DRB3*02:02	2	223	238	16	NetMHCIIpan	YNVLVLNPSVAATLGF	0.02	0.02	-	-	-	-	-	-
HLA-DRB1*01:01	1	554	568	15	Consensus (comb.lib./smm/nn)	GLNFAYLTAYQATVC	0.03	0.03	FAYLTAYQA	0.01	0.01	0.01	FAYLTAYQA	10.00	1.10	1.10
HLA-DRB1*01:01	1	555	569	15	Consensus (comb.lib./smm/nn)	LNFAYLTAYQATVCA	0.03	0.03	FAYLTAYQA	0.01	0.01	0.01	FAYLTAYQA	10.00	1.10	1.10
HLA-DRB1*01:01	1	556	570	15	Consensus (comb.lib./smm/nn)	NFAYLTAYQATVCAR	0.03	0.03	FAYLTAYQA	0.01	0.01	0.01	FAYLTAYQA	27.00	4.60	4.60
HLA-DRB1*01:01	1	553	567	15	Consensus (comb.lib./smm/nn)	QGLNFAYLTAYQATV	0.03	0.03	FAYLTAYQA	0.01	0.01	0.01	FAYLTAYQA	9.00	0.91	0.91
HLA-DRB3*02:02	1	220	236	17	NetMHCIIpan	AQGYNVLVLNPSVAATL	0.03	0.05	-	-	-	-	-	-
HLA-DRB3*02:02	2	220	236	17	NetMHCIIpan	AQGYNVLVLNPSVAATL	0.03	0.05	-	-	-	-	-	-
HLA-DRB3*02:02	1	222	238	17	NetMHCIIpan	GYNVLVLNPSVAATLGF	0.03	0.05	-	-	-	-	-	-
HLA-DRB3*02:02	2	222	238	17	NetMHCIIpan	GYNVLVLNPSVAATLGF	0.03	0.05	-	-	-	-	-	-
HLA-DRB3*02:02	1	224	239	16	NetMHCIIpan	NVLVLNPSVAATLGFG	0.04	0.05	-	-	-	-	-	-
HLA-DRB3*02:02	2	224	239	16	NetMHCIIpan	NVLVLNPSVAATLGFS	0.04	0.05	-	-	-	-	-	-
HLA-DRB3*02:02	1	221	237	17	NetMHCIIpan	QGYNVLVLNPSVAATLG	0.03	0.05	-	-	-	-	-	-
HLA-DRB3*02:02	2	221	237	17	NetMHCIIpan	QGYNVLVLNPSVAATLG	0.03	0.05	-	-	-	-	-	-

Figure 32: MHC-II epitope prediction (a snapshot).

5.4.2 Filtering MHC-I and MHC-II epitopes:

All 119 MHC-I and 436 MHC-II epitopes were filtered with the parameters mentioned in 6.6.2.

Antigenicity was checked by the VaxiJen 2.0 server.

Allergenicity was checked by the AllerTop V.-2 server.

Toxicity was checked by the ToxinPred server.

Human homology was checked with NCBI protein Blast (E value >2).

IFN-γ secretion capability was predicted by the IFN-Epitope server

Figure 33: Antigenicity prediction by VaxiJen 2.0 server (a snapshot).

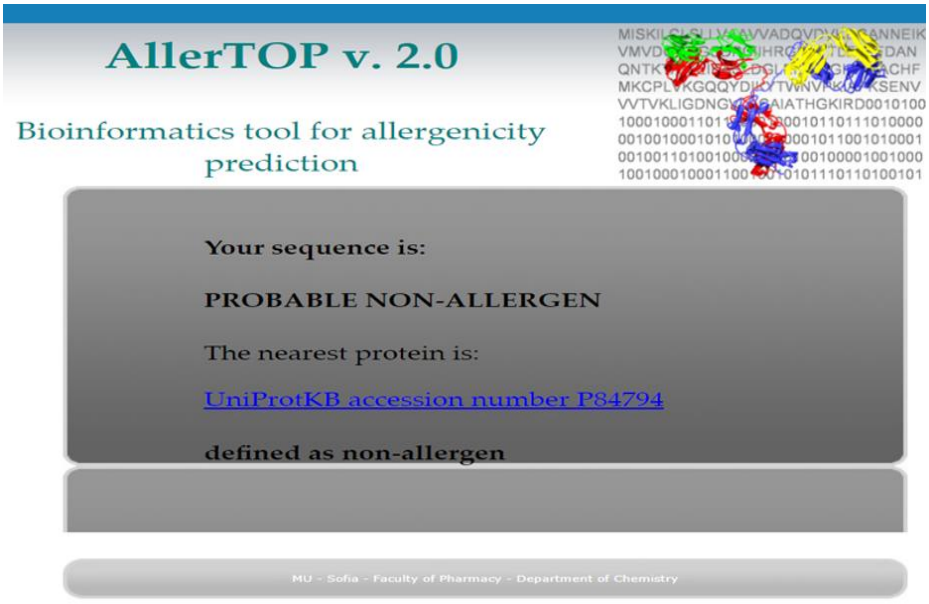

Figure 34: Allergenicity prediction by AllerTop v2.0 server (a snapshot).

Original Peptide								
Peptide Sequence	**Mutation Position**	**SVM score**	**Prediction**	**Hydrophobicity**	**Hydropathicity**	**Hydrophilicity**	**Charge**	**Mol wt**
AETGVRLRAY	No Mutation	-1.02	Non-Toxin	-0.26	-0.33	0.20	1.00	1135.42
Mutant Peptides								
CETGVRLRAY	1	-0.78	Non-Toxin	-0.28	-0.26	0.15	1.00	1167.48
DETGVRLRAY	1	-1.07	Non-Toxin	-0.35	-0.86	0.55	0.00	1179.43
EETGVRLRAY	1	-0.98	Non-Toxin	-0.34	-0.86	0.55	0.00	1193.46
FETGVRLRAY	1	-1.19	Non-Toxin	-0.22	-0.23	0.00	1.00	1211.52
GETGVRLRAY	1	-1.31	Non-Toxin	-0.27	-0.55	0.25	1.00	1121.40
HETGVRLRAY	1	-1.16	Non-Toxin	-0.32	-0.83	0.20	1.50	1201.49
IETGVRLRAY	1	-1.11	Non-Toxin	-0.21	-0.06	0.07	1.00	1177.51
KETGVRLRAY	1	-1.18	Non-Toxin	-0.39	-0.90	0.55	2.00	1192.52
LETGVRLRAY	1	-1.17	Non-Toxin	-0.23	-0.13	0.07	1.00	1177.51
METGVRLRAY	1	-1.29	Non-Toxin	-0.26	-0.32	0.12	1.00	1195.54
NETGVRLRAY	1	-1.13	Non-Toxin	-0.35	-0.86	0.27	1.00	1178.45
PETGVRLRAY	1	-1.23	Non-Toxin	-0.29	-0.67	0.25	1.00	1161.46
QETGVRLRAY	1	-1.12	Non-Toxin	-0.35	-0.86	0.27	1.00	1192.48
RETGVRLRAY	1	-0.88	Non-Toxin	-0.46	-0.96	0.55	2.00	1220.53
SETGVRLRAY	1	-1.14	Non-Toxin	-0.31	-0.59	0.28	1.00	1151.42
TETGVRLRAY	1	-1.25	Non-Toxin	-0.30	-0.58	0.21	1.00	1165.45
VETGVRLRAY	1	-1.15	Non-Toxin	-0.23	-0.09	0.10	1.00	1163.48
WETGVRLRAY	1	-1.03	Non-Toxin	-0.24	-0.60	-0.09	1.00	1250.56
YETGVRLRAY	1	-1.19	Non-Toxin	-0.28	-0.64	0.02	1.00	1227.52
AATGVRLRAY	2	-1.24	Non-Toxin	-0.17	0.20	-0.15	2.00	1077.38
ACTGVRLRAY	2	-0.68	Non-Toxin	-0.19	0.27	-0.20	2.00	1109.44
ADTGVRLRAY	2	-0.95	Non-Toxin	-0.27	-0.33	0.20	1.00	1121.39
AFTGVRLRAY	2	-1.35	Non-Toxin	-0.13	0.30	-0.35	2.00	1153.48
AGTGVRLRAY	2	-1.03	Non-Toxin	-0.18	-0.02	-0.10	2.00	1063.36

Figure 35: toxicity was predicted by ToxinPred2 server (a snapshot).

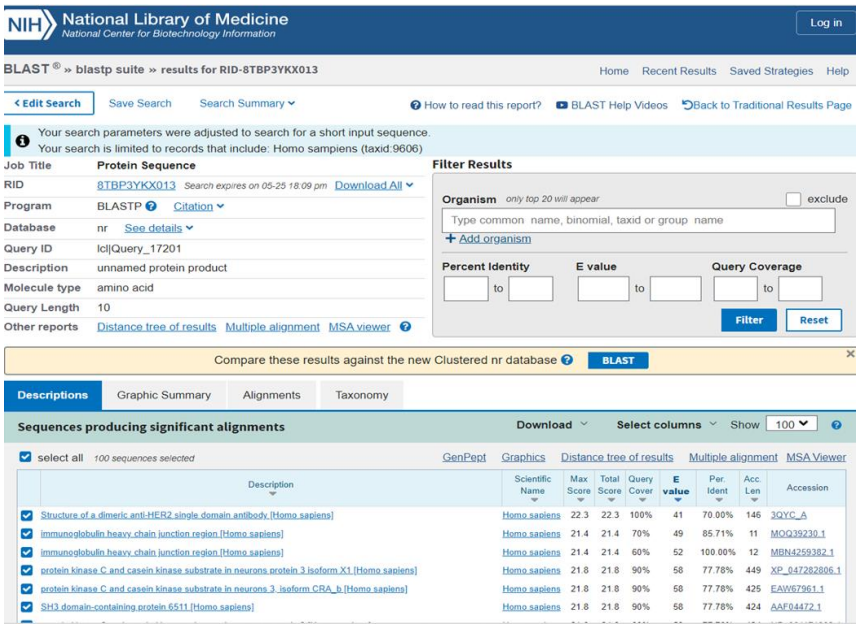

Figure 36: Human homology was predicted by NCBI protein Blast (E-value > 2).

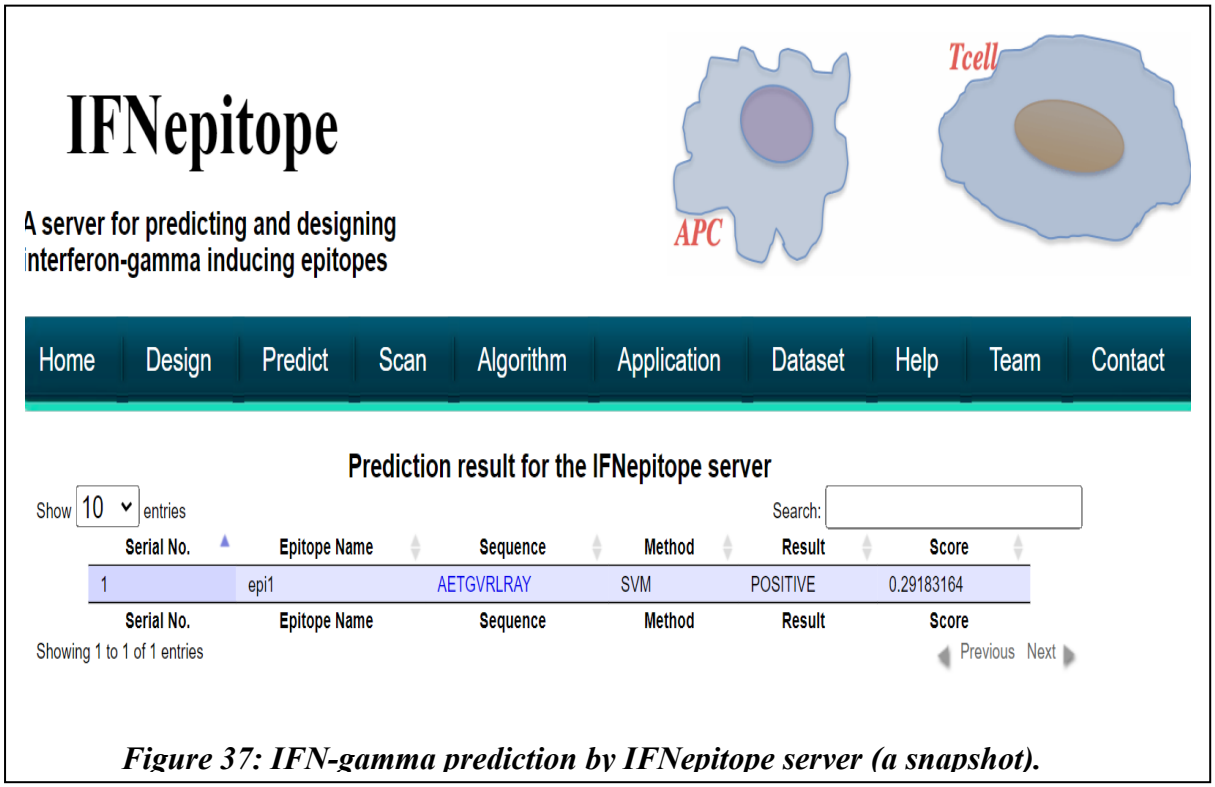

Figure 37: IFN-gamma prediction by IFNepitope server (a snapshot).

Two additional parameters (IL-4 and IL-10 secretion capability) for MHC-II epitopes were checked with IL-4Pred and IL-10Pred respectively-

Original Peptide								
Peptide Sequence	Mutation Position	SVM score	Prediction	Hydrophobicity	Hydropathicity	Hydrophilicity	Charge	Mol wt
AETGVRLRAY	No Mutation	0.30	IL4-inducer	-0.26	-0.33	0.20	1.00	1135.42
Mutant Peptides								
CETGVRLRAY	1	0.28	IL4 inducer	-0.28	-0.26	0.15	1.00	1167.48
DETGVRLRAY	1	0.30	IL4 inducer	-0.35	-0.86	0.55	0.00	1179.43
EETGVRLRAY	1	0.26	IL4 inducer	-0.34	-0.86	0.55	0.00	1193.46
FETGVRLRAY	1	0.31	IL4 inducer	-0.22	-0.23	0.00	1.00	1211.52
GETGVRLRAY	1	0.30	IL4 inducer	-0.27	-0.55	0.25	1.00	1121.40
HETGVRLRAY	1	0.22	IL4 inducer	-0.32	-0.83	0.20	1.50	1201.49
IETGVRLRAY	1	0.33	IL4 inducer	-0.21	-0.06	0.07	1.00	1177.51
KETGVRLRAY	1	0.30	IL4 inducer	-0.39	-0.90	0.55	2.00	1192.52
LETGVRLRAY	1	0.36	IL4 inducer	-0.23	-0.13	0.07	1.00	1177.51
METGVRLRAY	1	0.29	IL4 inducer	-0.26	-0.32	0.12	1.00	1195.54
NETGVRLRAY	1	0.32	IL4 inducer	-0.35	-0.86	0.27	1.00	1178.45
PETGVRLRAY	1	0.31	IL4 inducer	-0.29	-0.67	0.25	1.00	1161.46
QETGVRLRAY	1	0.29	IL4 inducer	-0.35	-0.86	0.27	1.00	1192.48
RETGVRLRAY	1	0.20	IL4 inducer	-0.46	-0.96	0.55	2.00	1220.53

Figure 38:IL4 induction prediction by IL-4Pred server.

Result Page of Predict

This page is the output of the Prediction of the IL10 inducers among the Query Sequences given by the user. The table below is a provides the details of the Query peptides given as input by the user with first column displaying the Staring Residue Position, second column for the sequence of the peptide, the third column providing the score given by the Machine Learning Algorithm according to the Prediction Model and the fourth column providing the Prediction whether the peptide is an Inducer or a Non-Inducer determined by the condition whether the Score is greater or less than the user defined threshold (in case of SVM) and whetehr probability is greater than-equal to or less than threshold probability in case of Random Forest method.

ID	Seq	Score	Prediction	Hydrophobicity	Hydropathicity	Hydrophilicity	Charge	Mol wt
1	TGLTHIDAHFLSQTKQ	0.65101497	IL10 inducer	-0.12	-0.46	-0.24	1.00	1797.25

Showing 1 to 1 of 1 rows

Figure 39: IL10 induction prediction by IL10 server.

After filtering based on positive antigenicity, negative allergenicity, negative toxicity, non-human homolog, and capability of IFN-γ secretion in the case of MHC-I and two additional

Immune cell type	Epitope	Start	End	Score
MHC class I	VPTGASLTY	259	267	0.993065
MHC class I	STASLLSPR	122	130	0.969748
MHC class I	TPPAVPQSY	189	197	0.961558
MHC class I	AETGVRLRAY	507	516	0.959918
MHC class I	AETTVRLRAY	507	516	0.943017
MHC class I	QPAETTVRL	505	513	0.91133
MHC class I	QPAETGVRL	505	513	0.9105
MHC class I	GSEGEIPFY	342	350	0.907844
MHC class I	KPTLTGPTPL	589	598	0.9056
MHC class I	KQQGLNFSY	551	559	0.893065
MHC class I	DSTASLLSPR	121	130	0.879576
MHC class I	EAVSRSQRR	454	462	0.878967
MHC class I	VEQYVDFSL	432	440	0.875826
MHC class I	ITAYAQQTR	3	11	0.868095
MHC class I	STASLLSPR	122	130	0.857386
MHC class I	ETTVRLRAY	508	516	0.855118
MHC class I	SQTKQQGLNF	548	557	0.843558
MHC class I	EADVIPARR	110	118	0.843558
MHC class I	STASLLSPR	122	130	0.835318
MHC class I	QPAETTVRL	505	513	0.8312
MHC class I	LAGVKHPAL	64	72	0.755883
MHC class I	DAVSRSQRR	454	462	0.726364
MHC class I	VTTGAKLTY	259	267	0.712832
MHC class I	VTTGAKLTY	259	267	0.690888
MHC class I	DAVSRSQRR	454	462	0.65671
MHC class I	QPAETTVRL	505	513	0.64994
MHC class I	STASLLSPR	122	130	0.642442
MHC class I	STPPAVPQSY	188	197	0.634157
MHC class I	VPAAYVAQGY	214	223	0.612164
MHC class I	KQQGLNFSY	551	559	0.602956
MHC class I	YDLQPAETTV	502	511	0.596536
MHC class I	LGFGSFMSR	236	244	0.592196
MHC class I	QPAETTVRL	505	513	0.584573
MHC class I	TLGFGSFMSR	235	244	0.573562
MHC class I	CECYDAGCSW	492	501	0.567053
MHC class I	DSTASLLSPR	121	130	0.553942

Figure 40: Filtered epitopes of MHC-I.

parameters (IL4 and IL10) for MHC-II epitopes, a total of 53 epitopes (MHC-I and II) were predicted.

Immune cell type	Epitope	Start	End
MHC Class II	AATLGFGSYMSRAYG	223	238
MHC Class II	GLNFSYLAAYQATVC	554	568
MHC Class II	LNFSYLAAYQATVCA	555	563
MHC Class II	QGLNFSYLAAYQATV	553	567
MHC Class II	QQGLNFSYLAAYQAT	552	566
MHC Class II	QGLNFSYLAAYQATVC	553	568
MHC Class II	KCLVRLKPTLTGPTPLLY	583	600
MHC Class II	LNPSVAATLGFGSFMS	228	243
MHC Class II	VAATLGFGSFMSRAYG	232	247
MHC Class II	PSVAATLGFGSFMSRAY	230	246
MHC Class II	SVAATLGFGSFMSRAYG	231	247
MHC Class II	FTGLTHIDAHFLSQ	536	549
MHC Class II	TGLTHIDAHFLSQTK	537	551
MHC Class II	GLTHIDAHFLSQTK	538	551
MHC Class II	TGLTHIDAHFLSQTKQ	537	552
MHC Class II	WDEMWKCLVRLKPTL	578	592

Figure 41: Filtered epitopes of MHC-II.

5.4.3 Conservancy analysis of filtered MHC-I and MHC-II epitopes:

After conservancy analysis 11 MHC-I epitopes and 6 MHC-II epitopes were found. **(Tables 14 and 15)**

Table 14: Conservancy analysis of MHC-I epitopes.

Sl. Number	Epitope sequence	Epitope length	Position	Antigenic score	% of protein sequence matches at identity <= 100%	Minimum identity
1	AETTVRLRAY	10	507-516	0.8876	100.00% (485/485)	80.00%
2	CECYDAGCSW	10	492-501	0.6717	100.00% (485/485)	80.00%
3	DAVSRSQRR	9	454-462	0.8561	100.00% (485/485)	88.89%
4	DSTASLLSPR	10	121-130	0.8536	100.00% (485/485)	90.00%
5	ITAYAQQTR	9	3-11	0.7046	100.00% (485/485)	88.89%
6	KPTLTGPTPL	10	589-598	0.5967	100.00% (485/485)	80.00%
7	SQTKQQGLNF	10	548-557	1.2047	100.00% (485/485)	90.00%
8	STASLLSPR	9	122-130	0.921	100.00% (485/485)	88.89%
9	STPPAVPQSY	10	188-197	0.4038	100.00% (485/485)	80.00%
10	VEQYVDFSL	9	432-440	1.2687	100.00% (485/485)	88.89%
11	VPAAYVAQGY	10	214-223	0.6642	100.00% (485/485)	100.00%

Table 15: conservancy analysis of MHC-II epitopes.

sl. Number	Epitope sequence	Epitope length	Position	Antigenic score	% of protein sequence matches at identity <= 100%	Minimum identity
1	FTGLTHIDAHFLSQ	14	536-549	0.7548	100.00% (485/485)	85.71%
2	FTGLTHIDAHFLSQTK	16	536-551	0.9516	100.00% (485/485)	87.50%
3	GLTHIDAHFLSQTK	14	538-551	1.0645	100.00% (485/485)	85.71%
4	TGLTHIDAHFLSQTK	15	537-551	1.0439	100.00% (485/485)	86.67%
5	TGLTHIDAHFLSQTKQ	16	537-552	1.0391	100.00% (485/485)	87.50%
6	WDEMWKCLVRLKPTL	15	578-592	0.4131	100.00% (485/485)	80.00%

5.5 Validation of Epitopes:

5.5.1 *In-silico* validation:

All MHC-I and MHC-II epitopes were docked to the appropriate MHC-I and II alleles, and the best model was refined. Each MHC-I epitope was docked with all MHC-I alleles. The ligand Root Mean Square Deviation (RMSD) values were determined during refinement studies and examined for satisfactory docking prediction. Each refinement (epitope + allele) produced ten alternative models with ten distinct RMSDs. To investigate how each epitope interacted with each allele, the median RMSD value was obtained from ten different models. The median RMSD values derived from several epitope-allele combinations were presented in the yarr-pirate-plot package in R studio to analyse which epitope interacts best with different types of MHC-I alleles.

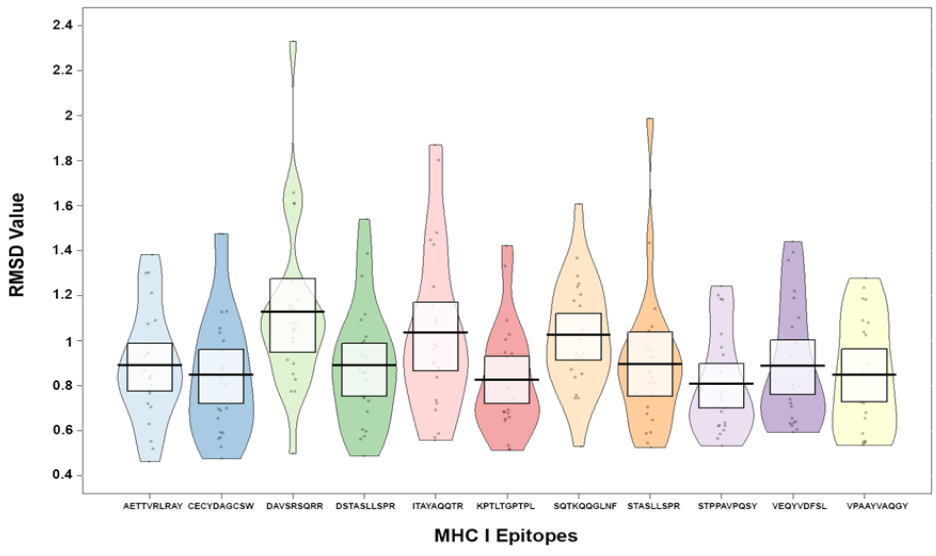

Figure 42: A violin plot has been generated with Median RMSD values found by docking refinement analysis between each MHC-I allele chain with each epitope.

Similarly, for MHC-II alleles, each chain of the MHC-II molecule was docked with each MHC-II epitope before refining the best model. The median RMSD values were obtained as described above and shown.

A total of 2530 potential models were created using 11 MHC-I epitopes and 23 MHC-I alleles. In the instance of MHC-I epitopes, 253 median RMSD values were calculated from various potential epitope + allele combinations (11 epitopes x 23 alleles). In most cases of MHC-I epitopes, RMSD values between epitope and allele combinations are less than 2Å. The median RMSD value for MHC-I epitopes with various alleles is also within 1.5 Å, showing good interactions with MHC-I epitopes with MHC-I alleles (**Figure 42**).

A total of 720 models were produced using 12 distinct MHC-II allele sub-chains and 6 MHC-II epitopes. A total of 72 median RMSD values were calculated from 720 distinct models. Although the median RMSD value with different sub-chains of MHC-II alleles is within 2Å, there are certain combinations in which the RMSD value is found to be larger than 2Å. This implies that MHC-II molecules are more confined toward any epitope than those of MHC-I molecules (**Figure 43**).

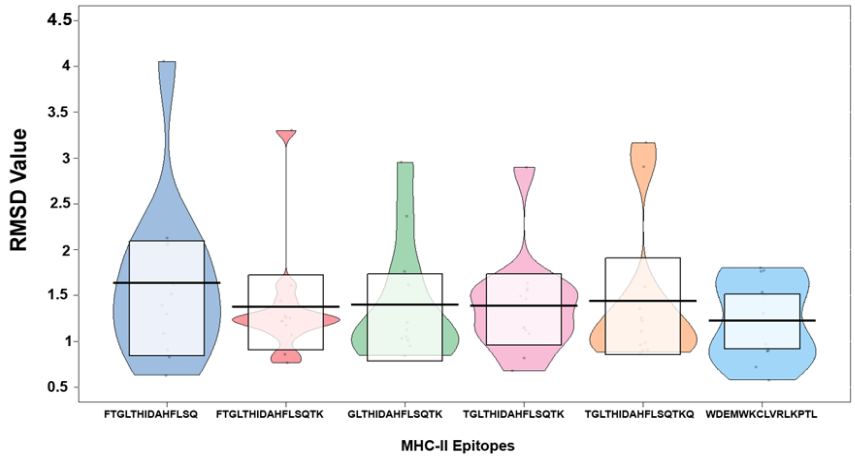

Figure 43: A violin plot has been generated with Median RMSD values found by docking refinement analysis between each sub-chain of MHC-II allele with each epitope.

$_{507}$AETTVRLRAY$_{516}$, $_{432}$VEQYVDFSL$_{440}$, $_{492}$CECYDAGCSW$_{501}$, $_{581}$KPTLTGPTPL$_{598}$, $_{188}$STPPAVPQSY197, and $_{214}$VPAAYVAQGY$_{223}$ among MHC-I epitopes shows median RMSD score <1.6, indicates very good interactions with diverse MHC-I alleles.

MHC-II epitope $_{578}$WDEMWKCLVRLKPTL$_{592}$ has a median RMSD score of <1.6, indicating it can bind to various allelic chains of MHC-II molecules. However, $_{536}$FTGLTHIDAHFLSQTK$_{549}$, $_{538}$GLTHIDAHFLSQTK$_{551}$, $_{537}$TGLTHIDAHFLSQTK$_{551}$, and $_{537}$TGLTHIDAHFLSQTKQ$_{552}$ also demonstrated excellent interaction, with most values falling within 2Å.

5.5.2 Regression analysis between RMSD value and antigenic score:

The 'VaxiJen' server's antigenic score has also been shown to predict RMSD values. However, more in-depth studies with wet lab experiments are needed to confirm those. Regression analysis has been used to determine if the 'RMSD values' of epitopes and 'Antigenic scores' of epitopes are depend on each other or not. Regression analysis was conducted with R packages. 'RMSD values' and 'Antigenic scores' of epitopes were used as dependent and independent variables or vice versa.

It has been found that 'RMSD value' and 'Antigenic score' can be a good predictor of each other. Details of analyses can be found below-

```
Call:
lm(formula = RMSD_Values ~ Antigenic_score)

Residuals:
     Min       1Q   Median       3Q      Max
-0.46723 -0.21350 -0.05001  0.15486  1.41086

Coefficients:
               Estimate Std. Error t-value Pr(>|t|)
(Intercept)     0.78271    0.06535  11.978  <2e-16 ***
Antigenic_score 0.16170    0.07631   2.119   0.0351 *
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 0.2949 on 251 degrees of freedom
Multiple R-squared:  0.01758,   Adjusted R-squared:  0.01366
F-statistic:  4.49 on 1 and 251 DF,  p-value: 0.03507
```

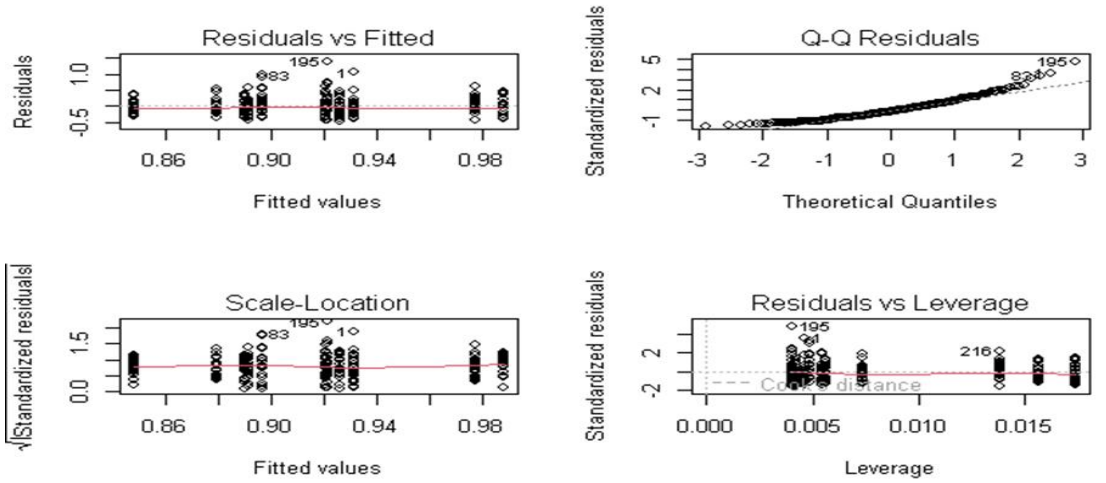

Figure 44: Regression analysis (RMSD values vs. Antigenic score).

MHC-I

Characteristic	N = 253[1]	p-value[2]
MHC-I Alleles		0.3
HLA_A_01_01	0.98 (0.84, 1.08)	
HLA_A_02_01	0.75 (0.56, 0.88)	
HLA_A_02_06	1.03 (0.84, 1.08)	
HLA_A_02_07	0.94 (0.89, 1.16)	
HLA_A_03_01	0.92 (0.80, 1.10)	
HLA_A_11_01	0.85 (0.68, 1.12)	
HLA_A_24_02	0.81 (0.72, 0.95)	
HLA_A_68_01	0.81 (0.66, 1.17)	
HLA_B_07_02	0.78 (0.71, 0.94)	
HLA_B_14_02	0.80 (0.76, 0.84)	
HLA_B_15_01	0.93 (0.71, 1.19)	
HLA_B_15_02	1.13 (0.89, 1.38)	
HLA_B_18_01	0.72 (0.64, 0.95)	
HLA_B_35_01	0.84 (0.67, 1.11)	
HLA_B_35_08	0.84 (0.71, 1.24)	
HLA_B_40_01	0.73 (0.63, 0.90)	
HLA_B_40_02	0.71 (0.62, 0.94)	
HLA_B_44_03	0.87 (0.72, 1.14)	
HLA_B_44_05	0.96 (0.90, 1.07)	
HLA_B_51_01	0.93 (0.74, 1.26)	
HLA_B_52_02	0.86 (0.77, 0.92)	
HLA_B_57_01	0.83 (0.70, 0.97)	
HLA_B_58_01	0.84 (0.74, 1.07)	
MHC-I epitopes		0.004
AETTVRLRAY	0.84 (0.74, 1.03)	
CECYDAGCSW	0.81 (0.62, 1.02)	
DAVSRSQRR	1.06 (0.91, 1.17)	
DSTASLLSPR	0.86 (0.71, 1.01)	
ITAYAQQTR	0.96 (0.79, 1.19)	
KPTLTGPTPL	0.74 (0.67, 0.94)	
SQTKQQGLNF	1.01 (0.86, 1.19)	
STASLLSPR	0.85 (0.69, 0.96)	
STPPAVPQSY	0.74 (0.62, 0.95)	
VEQYVDFSL	0.80 (0.65, 1.08)	
VPAAYVAQGY	0.85 (0.62, 1.06)	

[1] RMSD Values: Median (IQR)

[2] Kruskal-Wallis rank sum test

MHC-II

Characteristic	N = 72[1]	p-value[2]
MHC-II Alleleic subchains		0.003
HLA_DMA	0.90 (0.86, 1.04)	
HLA_DMB	1.43 (1.28, 1.92)	
HLA_DPA1	0.97 (0.87, 1.18)	
HLA_DPB1	1.94 (1.55, 2.70)	
HLA_DQ1	1.00 (0.86, 1.21)	
HLA_DQA1	1.22 (1.02, 1.29)	
HLA_DQB	1.68 (1.57, 1.77)	
HLA_DQB1	1.58 (1.24, 1.62)	
HLA_DRA1	1.00 (0.76, 1.18)	
HLA_DRB1	1.13 (1.00, 1.52)	
HLA_DRB3	1.23 (1.03, 1.67)	
HLA_DRB5	1.05 (0.94, 1.36)	
MHC-II Epitopes		>0.9
FTGLTHIDAHFLSQ	1.46 (1.04, 2.06)	
FTGLTHIDAHFLSQTK	1.24 (1.15, 1.32)	
GLTHIDAHFLSQTK	1.09 (1.00, 1.65)	
TGLTHIDAHFLSQTK	1.31 (1.11, 1.56)	
TGLTHIDAHFLSQTKQ	1.17 (0.95, 1.42)	
WDEMWKCLVRLKPTL	1.14 (0.90, 1.59)	

[1] RMSD Value: Median (IQR)

[2] Kruskal-Wallis rank sum test

Table 16: RMSD analysis of MHC-I and II epitopes with various HLA alleles.

5.6 *In-vitro* analysis:

5.6.1 CFDA cell proliferation assay:

To successfully validate the in-silico technique, the 11 best epitopes (MHC-I + MHC-II) listed above were synthesised and purified. These were then evaluated for their potential to stimulate the proliferation of PBMC T-cells obtained from ten healthy participants. To assess cell proliferation, the mean fluorescence intensity (MFI) was recorded in each test well of a 24-well plate and compared to the negative control (pulsed with PBS instead of peptides). MFI of unlabeled cells was obtained to assess cellular autofluorescence. To minimise undesired immunological reactions, PBMCs from various individuals were grown separately. For all cases, MFI values were displayed and statistically compared using pulsed peptide vs no peptide (negative control), no peptide versus unlabelled, and unlabelled versus pulsed peptide using Wilcoxon sign rank test and Kruskal-Walis test in R-studio (R version 3.2). Significant changes were recognised at a p-value <0.05. It was observed that the best-predicted epitopes might trigger cell growth. In every example, MFI was considerably lower in epitope-pulsed PBMC than in PBS-pulsed PBMC. MHC-I epitopes were observed to stimulate cell proliferation more strongly than MHC-II epitopes. Overall, the MFI decrease results showed PBMC expansion, emphasising the promise of *in-silico* techniques for epitope prediction (**Figure 45** and **Figure 46**).

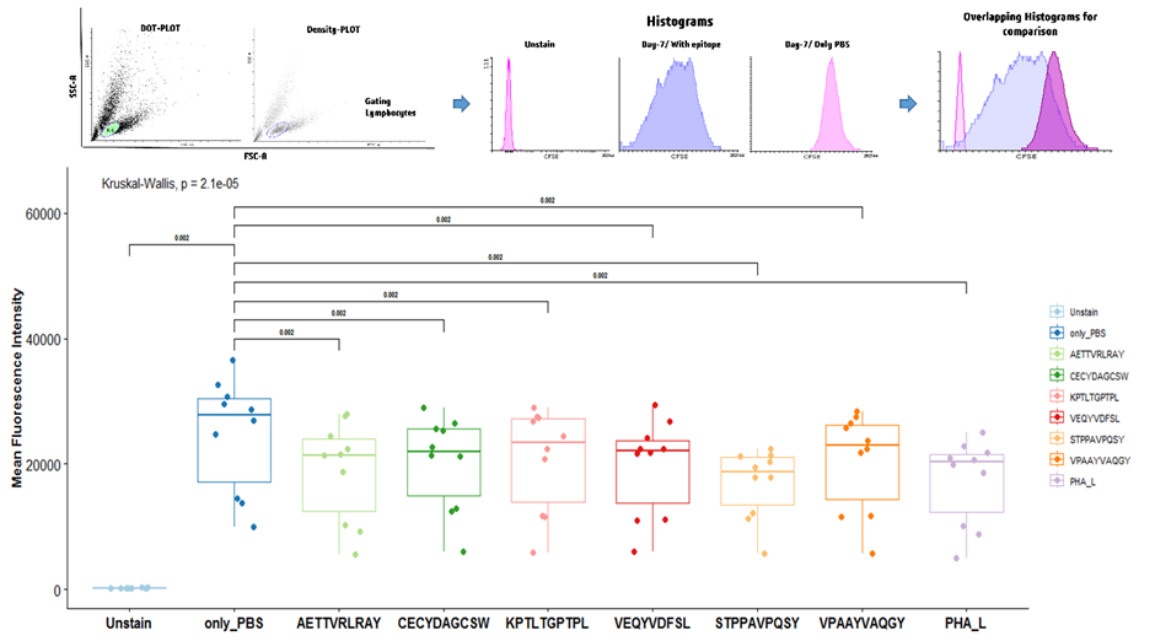

Figure 45: CFDA-SE cell proliferation assay with MHC-I peptides. CFDA-SE tagged PBMC were pulsed with selected MHC-I epitopes and incubated for 7 days. After 7 days, Mean Fluorescent Intensity (MFI) of 20,000 lymphocyte gated events, based on scatter parameters of size and granularity was acquired. "only_PBS" refers to PBMC pulsed with PBS (Negative control), "PHA_L" represents PBMC pulsed with Phytohemagglutinin (positive control), "Unstain" refers to PBMC not being stained with CFDA-SE, which gives an idea about cellular autofluorescence. Reference images of lymphocyte gating and CFSE histograms of "Unstain", "Epitopes" and "Only PBS" (Negative control). Overlapping histograms indicate a decrease in MFI (Top). MFI values found from all volunteers' PBMC pulsing experiments were grouped and plotted. In all cases of epitope pulsed PBMC from different volunteers, MFI significantly decreases than negative control. The MFI Value of "Unstain" clearly indicates that cellular autofluorescence did not interfere with the result. (Bottom) [Wilcoxon sign rank test was used to compare between Negative control (reference group) and other groups. Kruskal-Walis test was used to compare between all groups.]

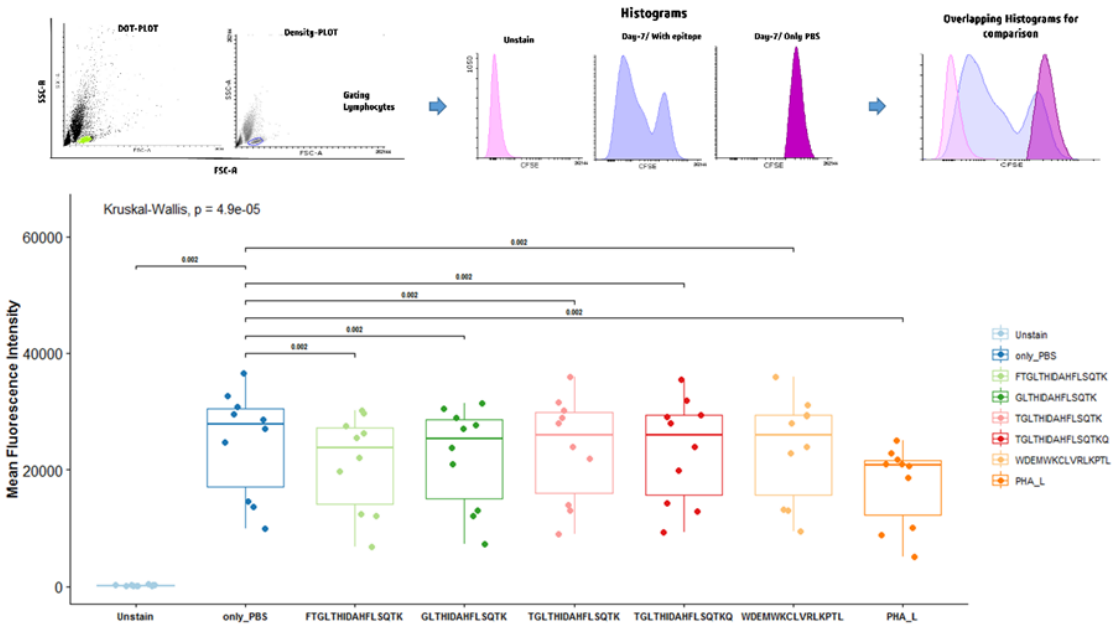

Figure 46: CFDA-SE cell proliferation assay with MHC-II peptides. CFDA-SE tagged PBMC were pulsed with selected MHC-II epitopes and incubated for 7 days. After 7 days, Mean Fluorescent Intensity (MFI) of 20,000 lymphocyte gated events, based on scatter parameters of size and granularity was acquired. "only_PBS" refers to PBMC pulsed with PBS (Negative control), "PHA_L" represents PBMC pulsed with Phytohemagglutinin (positive control), "Unstain" refers to PBMC not being stained with CFDA-SE, which gives an idea about cellular autofluorescence. Reference images of lymphocyte gating and CFSE histograms of "Unstain", "Epitopes" and "Only PBS" (Negative control). Overlapping histograms indicate a decrease in MFI (Top). MFI values found from all volunteers' PBMC pulsing experiments were grouped and plotted. In all cases of epitope pulsed PBMC from different volunteers, MFI significantly decreases than negative control. The MFI Value of "Unstain" clearly indicates that cellular autofluorescence did not interfere with the result. (Bottom) [Wilcoxon sign rank test was used to compare between Negative control (reference group) and other groups. Kruskal-Walis test was used to compare between all groups.]

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5.6.2 Validation of epitopes by IFN-γ ELISA:

After 5 days of pulsing PBMC with MHC-I and MHC-II peptides (epitopes), the quantity of interferon-γ was measured using an IFN-γ ELISA. All selected MHC-I and MHC-II epitopes induced IFN-γ release. No IFN-γ was detected in the medium without PBMC (data not shown). MHC-I epitopes resulted in more IFN-γ production than MHC-II epitopes. Overall, the mean concentration of IFN-γ with MHC-I pulsed epitope was 1368pg/ml ± 150.70. In the case of MHC-II epitopes, the mean IFN-γ concentration was 1257pg/ml±64.07 (**Figure 47 and 48**).

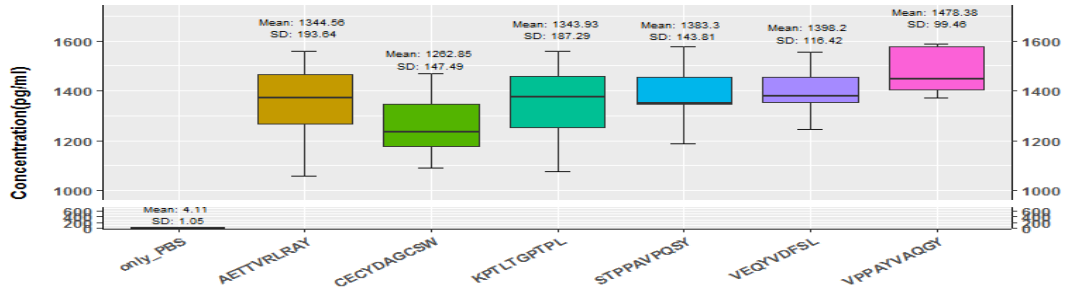


Figure 47: The IFN-γ ELISA test was used to measure the interferon-γ concentration after PBMC were pulsed for five days with selected MHC-I epitopes. IFN-γ was barely detected on PBS pulsed PBMC (only_PBS), which serves as the negative control. On the other hand, MHC-I epitopes showed a significant amount of IFN-γ expression.

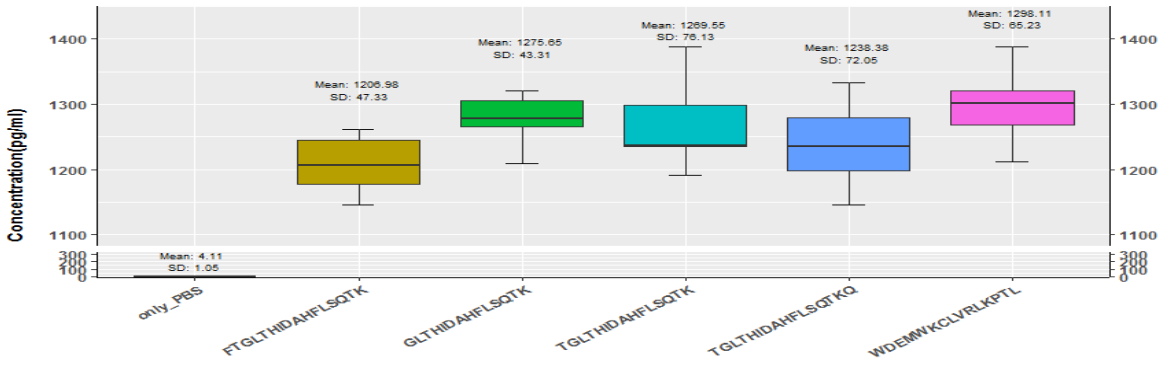


Figure 48: The IFN-γ ELISA test was used to measure the interferon-γ concentration after PBMC were pulsed for five days with selected MHC-II epitopes. IFN-γ was barely detected on PBS pulsed PBMC (only_PBS), which serves as the negative control. On the other hand, MHC-II epitopes showed a significant amount of IFN-γ expression.

5.7 Mutational analysis of epitopes with other HCV variants:

It was observed that, among these 11 epitopes, some of them were conserved in other genotypes and subtypes. Mutated epitopes were also checked for the basic four parameters antigenicity, allergenicity, toxicity and human homology. When mutated epitopes were found to pass basic four parameters then it was highlighted with green colour, otherwise, it was marked with red colour in the picture. Mutational analysis of epitopes shown below with other prevalent HCV genotypes of India. These are as follows-

5.7.1 Genotype 3b:

Genotype 3b

Conserved epitopes in MHC-I:

1. AETTVRLRAY
2. CECYDAGCSW
3. DAVSRSQRR
4. DSTASLLSPR
5. SQTKQQGLNF
6. STASLLSPR
7. VEQYVDFSL
8. VPPAYVAQGY

Conserved epitopes in MHC-II:

1. FTGLTHIDAHFLSQ
2. FTGLTHIDAHFLSQTK
3. GLTHIDAHFLSQTK
4. TGLTHIDAHFLSQTK
5. TGLTHIDAHFLSQTKQ

Variations found in 3b

MHC-I

Epitopes	Antigenic	Allargenic	Toxic	Human homolog
KPTLHGPTPL	No	No	No	No
ISAYAQQTR	Yes	No	No	No
STPPAVPESY	No	No	No	No

MHC-II

Epitopes	Antigenic	Allargenic	Toxic	Human homolog
WDEMWKCLIRLKPTL	Yes	No	No	No

Figure 49: Predicted epitope conservancy and variations found HCV subtype 3b. Variations in epitopes marked with a different colour with basic four parameter predictions. 'Red' colour indicates that the mutated epitopes failed to pass four parameter predictions, otherwise marked with green.

In the case of HCV genotype 3b, 8 MHC-I epitopes and 5 MHC-II epitopes were found to be conserved with HCV genotype 3a. MHC-I epitopes AETTVRLRAY, CECYDAGCSW, DAVSRSQRR, DSTASLLSPR, SQTKQQGLNF, STASLLSPR, VEQYVDFSL, and VPPAYVAQGY were found to conserved, whereas, KPTLTGPTPL (in HCV gen-3a) was

mutated in genotype 3b as KPTLHGPTPL, TAYAQQTR (in HCV gen-3a) was mutated in HCV genotype 3b as ISAYAQQTR, STPPAVPQSY (in HCV gen-3a) was mutated in genotype 3b as STPPAVPESY. Whereas, in the case of MHC-II epitopes only WDEMWKCLVRLKPTL (in gen-3a) was mutated to WDEMWKCLIRLKPTL (**Figure 49**).

5.7.2 Genotype 1b:

Genotype 1b

Conserved epitopes in MHC-I:

1. DAVSRSQRR

Conserved epitopes in MHC-II:

1. FTGLTHIDAHFLSQ
2. FTGLTHIDAHFLSQTK
3. GLTHIDAHFLSQTK
4. TGLTHIDAHFLSQTK
5. TGLTHIDAHFLSQTKQ

Variations found in 1b

MHC-I

Epitopes	Antigenic	Allargenic	Toxic	Human homolog
AETSVRLRAY	Yes	No	No	No
CECYDAGCAW	Yes	No	Yes	No
DSRGSLLSPR	Yes	Yes	No	No
ITAYSQQTR	Yes	Yes	No	No
KPTLHGPTPL	No	No	No	No
RGSLLSPR	Yes	Yes	No	No
SQTKQAGDNF	No	Yes	No	No
SSPPAVPQTF	No	No	No	No
VPAAYAAQGY	Yes	No	No	No
VTQTVDFSL	Yes	No	No	No

MHC-II

Epitopes	Antigenic	Allargenic	Toxic	Human homolog
WDQMWKCLIRLKPTL	Yes	No	No	No

Figure 50: Predicted epitope conservancy and variations found HCV subtype 1b. Variations in epitopes marked with a different colour with basic four parameter predictions. 'Red' colour indicates that the mutated epitopes failed to pass four parameter predictions, otherwise marked with green.

In the case of HCV genotype 1b, the only MHC-I epitope DAVSRSQRR was found to be identical to HCV genotype 3a. The rest of the MHC-I epitopes were mutated in single or multiple positions. In the case of MHC-II epitopes, out of the 6 epitopes, 5 epitopes were found to be identical with HCV Gen-3a. Only, the MHC-II epitope WDEMWKCLVRLKPTL (in HCV gen-3a) was mutated to WDQMWKCLIRLKPTL. Among all mutated epitopes only AETSVRLRAY, VPAAYAAQGY, VTQTVDFSL and WDQMWKCLIRLKPTL were found to pass the basic four parameters (**Figure 50**).

1.1.1 Genotype 1c:

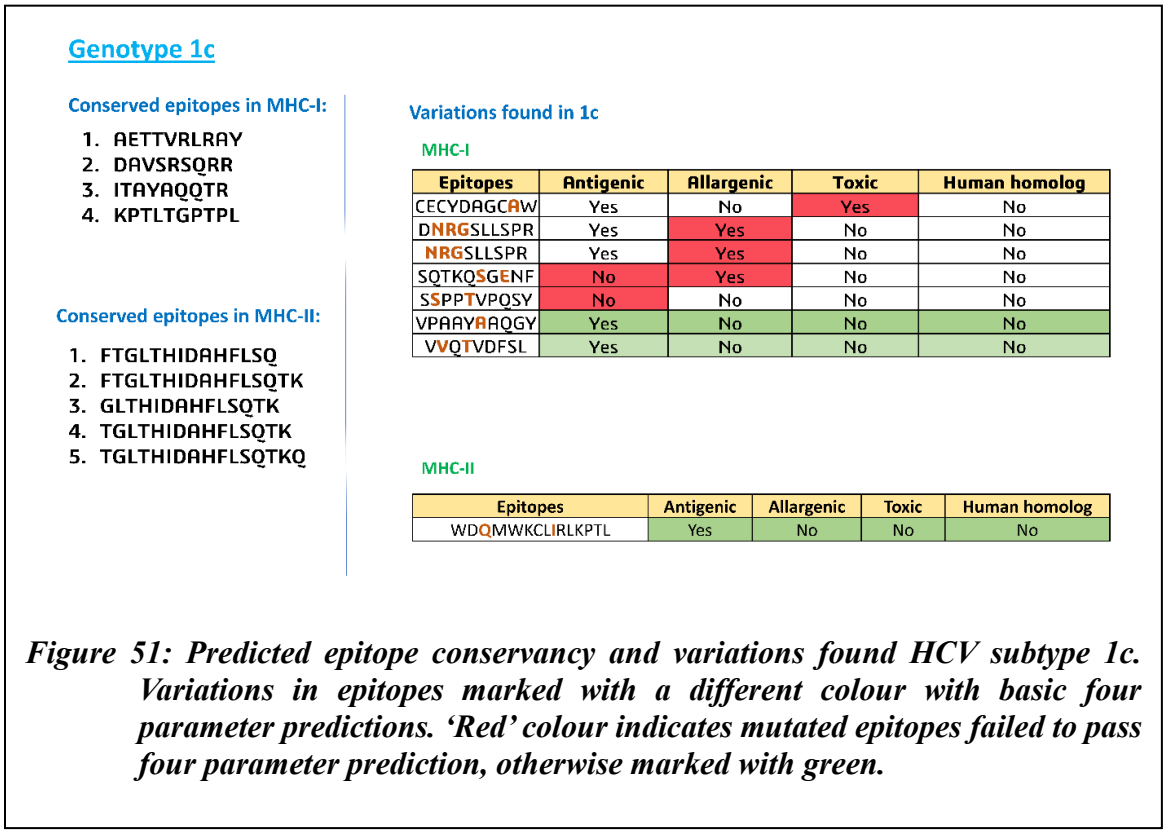


Figure 51: Predicted epitope conservancy and variations found HCV subtype 1c. Variations in epitopes marked with a different colour with basic four parameter predictions. 'Red' colour indicates mutated epitopes failed to pass four parameter prediction, otherwise marked with green.

In case of HCV genotype 1c, 4 MHC-I epitopes (AETTVRLRAY, DAVSRSQRR, ITAYAQQTR and KPTLTGPTPL) and 5 MHC-II epitopes (FTGLTHIDAHFLSQ, FTGLTHIDAHFLSQTK, GLTHIDAHFLSQTK, TGLTHIDAHFLSQTK, and TGLTHIDAHFLSQTKQ) were found to be identical with HCV gen-3a. Among all mutated epitopes VPAAYAAQGY, VTQTVDFSL and WDQMWKCLIRLKPTL were found to pass the basic four parameters (**Figure 51**).

5.7.3 Genotype 1a:

Genotype 1a

Conserved epitopes in MHC-I:

1. AETTVRLRAY
2. VPPAYVAQGY

Variations found in 1a

MHC-I

Epitopes	Antigenic	Allargenic	Toxic	Human homolog
CECYDAGCAW	Yes	No	Yes	No
DAVSRTQRR	Yes	No	No	No
DSRGSLLSPR	Yes	Yes	No	No
ITAYSQQTR	Yes	Yes	No	No
KPTLHGPTPL	No	No	No	No
RGSLLSPR	Yes	Yes	No	No
SQTKQAGDNF	No	Yes	No	No
SSPPAVPQTF	No	No	No	No
VTQTVDFSL	Yes	No	No	No

Conserved epitopes in MHC-II:

1. FTGLTHIDAHFLSQ
2. FTGLTHIDAHFLSQTK
3. GLTHIDAHFLSQTK
4. TGLTHIDAHFLSQTK
5. TGLTHIDAHFLSQTKQ

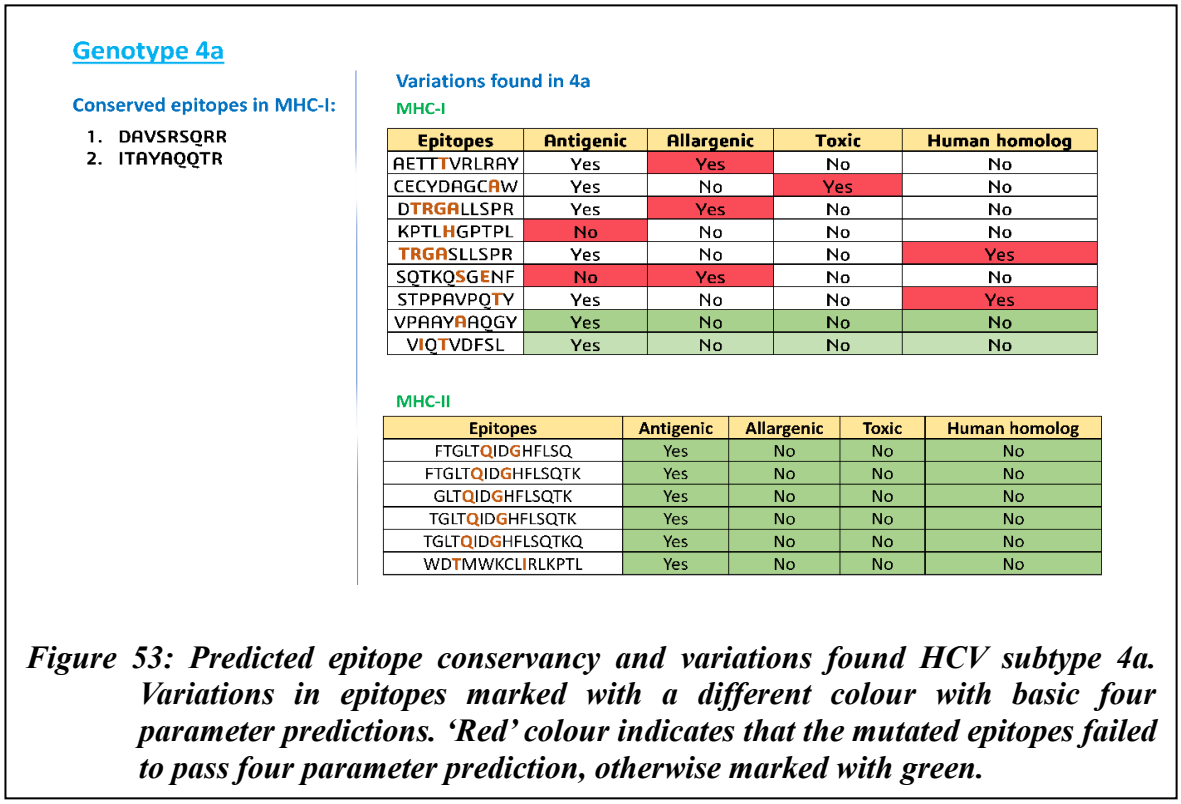
MHC-II

Epitopes	Antigenic	Allargenic	Toxic	Human homolog
WDQMWKCLIRLKPTL	Yes	No	No	No

Figure 52: Predicted epitope conservancy and variations found HCV subtype 1a. Variations in epitopes marked with a different colour with basic four parameter predictions. 'Red' colour indicates that the mutated epitopes failed to pass four parameter prediction, otherwise marked with green.

In the case of HCV genotype 1a, only 2 MHC-II epitopes were found to be identical with Gen-3a which were AETTVRLRAY and VPPAYVAQGY. Whereas, 5 MHC-II epitopes were found to be conserved in Genotype 1a. which are FTGLTHIDAHFLSQ, FTGLTHIDAHFLSQTK, GLTHIDAHFLSQTK, TGLTHIDAHFLSQTK and TGLTHIDAHFLSQTKQ. Among the mutated epitopes DAVSRTQRR, VTQTVDFSL and WDQMWKCLIRLKPTL were able to pass through the basic four parameters test (**Figure 52**).

5.7.4 Genotype 4a:



Genotype 4a

Conserved epitopes in MHC-I:

1. DAVSRSQRR
2. ITAYAQQTR

Variations found in 4a

MHC-I

Epitopes	Antigenic	Allargenic	Toxic	Human homolog
AETTTVRLRAY	Yes	Yes	No	No
CECYDAGCAW	Yes	No	Yes	No
DTRGALLSPR	Yes	Yes	No	No
KPTLHGPTPL	No	No	No	No
TRGASLLSPR	Yes	No	No	Yes
SQTKQSGENF	No	Yes	No	No
STPPAVPQTY	Yes	No	No	Yes
VPAAYAAQGY	Yes	No	No	No
VIQTVDFSL	Yes	No	No	No

MHC-II

Epitopes	Antigenic	Allargenic	Toxic	Human homolog
FTGLTQIDGHFLSQ	Yes	No	No	No
FTGLTQIDGHFLSQTK	Yes	No	No	No
GLTQIDGHFLSQTK	Yes	No	No	No
TGLTQIDGHFLSQTK	Yes	No	No	No
TGLTQIDGHFLSQTKQ	Yes	No	No	No
WDTMWKCLIRLKPTL	Yes	No	No	No

Figure 53: Predicted epitope conservancy and variations found HCV subtype 4a. Variations in epitopes marked with a different colour with basic four parameter predictions. 'Red' colour indicates that the mutated epitopes failed to pass four parameter prediction, otherwise marked with green.

In the case of HCV genotype 4a, only 2 MHC-I epitopes were found to be conserved. Those 2 MHC-I epitopes were DAVSRSQRR and ITAYAQQTR. However, No MHC-II epitopes were found to be identical to HCV genotype 3a. Among mutated epitopes VPAAYAAQGY, VIQTVDSL, FTGLTQIDGHFLSQ, FTGLTQIDGHFLSQTK, GLTQIDGHFLSQTK, TGLTQIDGHFLSQTK, FTGLTQIDGHFLSQTKQ and WDQMWKCLIRLKPTL were able to pass through basic four parameter tests (**Figure 53**).

5.7.5 Genotype 6h and 6n:

Genotype 6n and 6h

Conserved epitopes in MHC-I:

1. AETTVRLRAY
2. DAVSRSQRR

Conserved epitopes in MHC-II:

1. FTGLTHIDAHFLSQ
2. FTGLTHIDAHFLSQTK
3. GLTHIDAHFLSQTK
4. TGLTHIDAHFLSQTK
5. TGLTHIDAHFLSQTKQ

Variations found in 6n and 6h

MHC-I

Epitopes	Antigenic	Allargenic	Toxic	Human homolog
CEAYDAGCAW	Yes	Yes	No	No
DNRAALLSPR	Yes	Yes	No	No
ITAYTQQTR	Yes	No	No	No
KPMLTGPTPL	No	No	No	No
NRAALLSPR	Yes	Yes	No	No
SQTKQGGENF	No	Yes	No	No
TSPPAVPQSY	No	No	No	No
VPAAYAAQGY	Yes	No	No	No
VTQIVDFSL	Yes	Yes	No	No

MHC-II

Epitopes	Antigenic	Allargenic	Toxic	Human homolog
WDTMWKCLIRLKPML	Yes	Yes	No	No

Figure 54: Predicted epitope conservancy and variations found HCV subtype 6h and 6n. Variations in epitopes marked with a different colour with basic four parameter predictions. 'Red' colour indicates that the mutated epitopes failed to pass four parameter prediction, otherwise marked with green.

In the case of HCV genotypes 6h and 6n, 2 MHC-I epitopes, AETTVRLRAY and DAVSRSQRR, were found to be conserved. Whereas, In the case of MHC-II, 5 epitopes were Conserved with HCV gen-3a, those are FTGLTHIDAHFLSQ, FTGLTHIDAHFLSQTK, GLTHIDAHFLSQTK, TGLTHIDAHFLSQTK, and TGLTHIDAHFLSQTKQ. Among mutated epitopes ITAYTQQTR and VPAAYAAQGY were found to pass the basic four Parameters (**Figure 54**).

5.7.6 Genotype 6k:

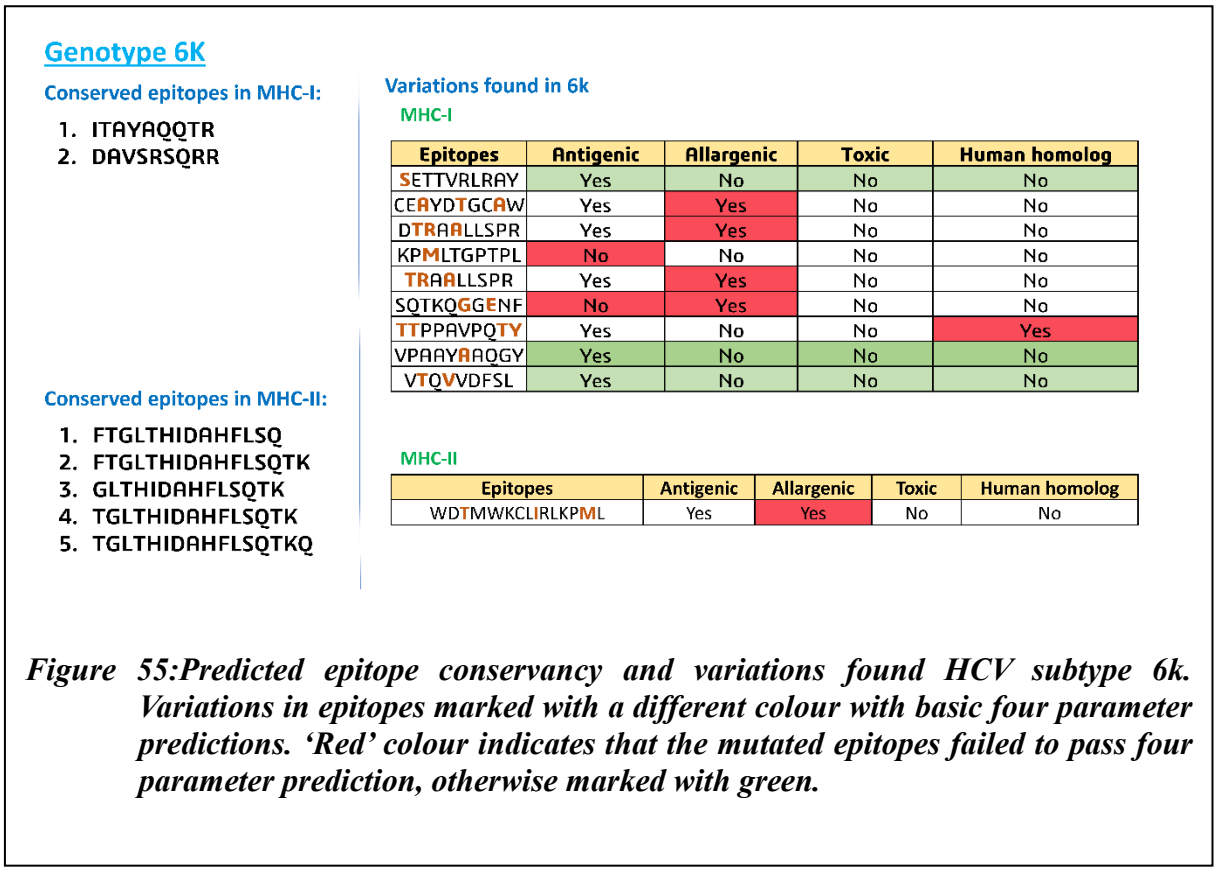


Figure 55:Predicted epitope conservancy and variations found HCV subtype 6k. Variations in epitopes marked with a different colour with basic four parameter predictions. 'Red' colour indicates that the mutated epitopes failed to pass four parameter prediction, otherwise marked with green.

In the case of HCV Genotype 6K, only 2 MHC-I epitopes (ITAYAQQTR and DAVSRSQRR) were identical to HCV genotype 3a. Whereas, 5 MHC –II epitopes (FTGLTHIDAHFLSQ, FTGLTHIDAHFLSQTK, GLTHIDAHFLSQTK, TGLTHIDAHFLSQTK and TGLTHIDAHFLSQTKQ) were found to be identical with genotype 3a. Among the mutated epitopes only SETTVRLRAY, VPAAYAAQGY and VTQVVDFSL were found to pass the basic four-parameter testing (**Figure 55**).

It was also found that at least 1 epitope is conserved in all prevalent HCV variants found in India (**Figure 56**).

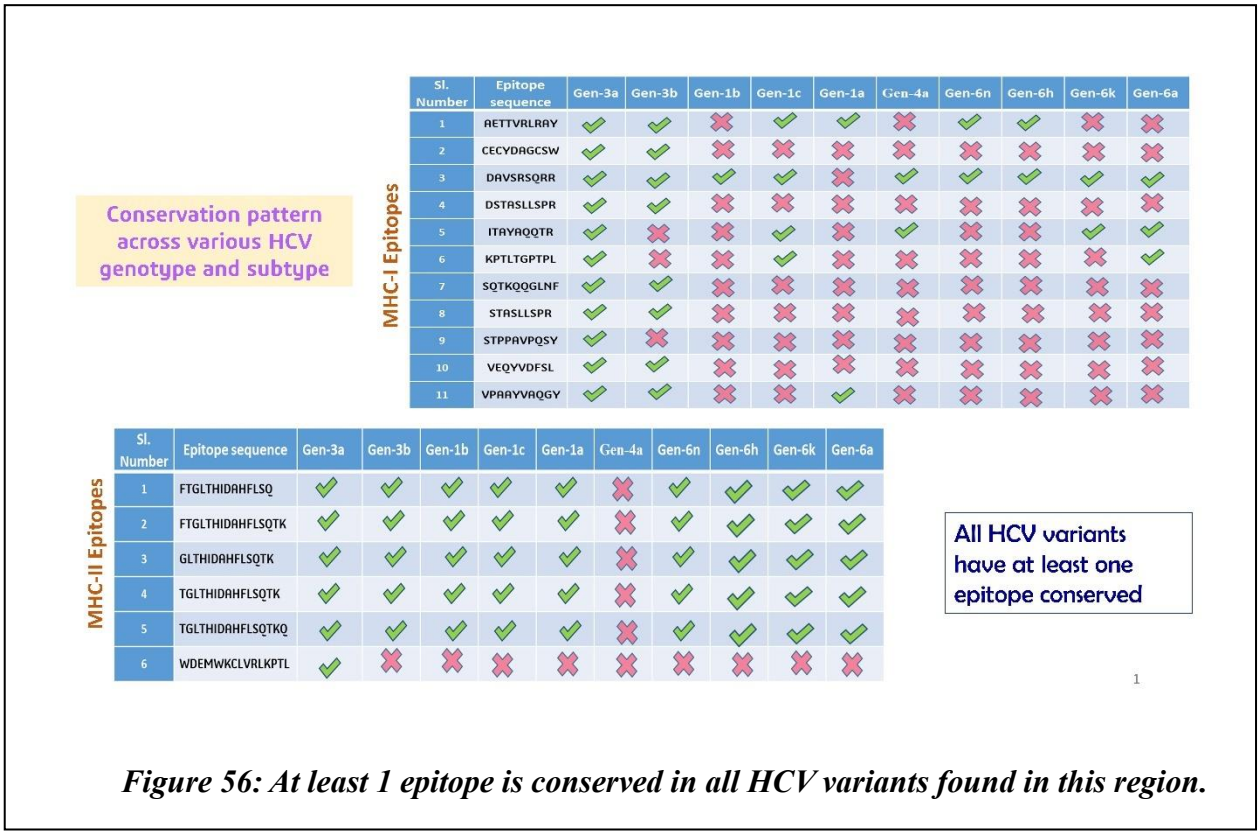


Figure 56: At least 1 epitope is conserved in all HCV variants found in this region.

Chapter 6

6 Discussion:

HCV is one of the world's major health problems. The World Health Organization has proposed the full elimination of HCV globally by 2030, and HCV infection has also been reduced from around 130 million (343) to 58 million (1) after the introduction of direct-acting antivirals (DAAs), but there are still various hindrances to achieving this goal. This is because of a lack of awareness of HCV among common people, access to NTA-based detection facilities in remote areas, the availability of DAAs in low-income countries, and the emergence of drug-resistant HCV strains (344). Therefore, awareness of HCV epidemiology must be continued for a better understanding of HCV disease dynamics. HCV often remains undiagnosed because, at the initial stages in most cases, it is asymptomatic. Thus, it is hard to determine new HCV infections in a population because it stays asymptomatic in the early stages. Due to a lack of medical awareness, low-income middle-class communities often ignore initial symptoms and HCV remains silent until significant liver damage occurs years later along with clinical signs and symptoms. In some cases, it becomes too late for the patient and ultimately leads to HCC and death.

There has been a revolution in HCV treatments in recent times. Current HCV therapy is very effective, especially pan-genotypic DAAs (345,346). Moreover, several nations have come up with an initiative to eradicate HCV (347). However, achieving HCV eradication presents numerous hurdles, such as greater availability and accessibility of pan-genotypic medicine, detection facilities, trained healthcare professionals, and so on. Apart from this, barriers exist between patients and individual healthcare providers as well as within the overall healthcare

system. Therefore, HCV treatment, prevention, and control activities require better epidemiologic data to guide control programs and enhance cost-effectiveness for healthcare systems. More extensive control and awareness programs as well as cost-effective therapy development and screening systems are needed at the global level to battle against HCV infection (345).

India contributes a significant part of global HCV infection. The HCV prevalence in India may vary from 0.5% to 1.5%, which lies in the intermediate range of HCV infection. Generally, higher in the North-Eastern part, tribal population, and North-west Punjab areas which are also identified as HCV hotspots of India (23). However, being an overpopulated country 0.5-1.5% contributes a significant amount of HCV burden towards the global HCV burden. Near about 12-18 million people are thought to be infected with HCV in India (237). Yet, it has been recognised that population-based HCV studies are rare, there were very few studies which discussed HCV prevalence in high-risk group populations in India (23).

High-risk groups are very prone to HCV infection. However, there are very limited studies that explore HCV epidemiology in these groups. Therefore, the first objective of this study has been set to analyse HCV viremia in different HCV-infected population groups in the eastern part of India and HCV-related disease complications (pathogenesis) among them. To accomplish this objective a total of 661 anti-HCV positive samples were collected along with complete clinical data from patient groups like Thalassemia, Chronic Kidney Disease (CKD), people who inject drugs ((PWIDs), and so on. Out of 661 samples, 403 samples were HCV RNA positive. Therefore, it can be deduced that overall viremia was observed in 60.96%. The major population group found in this study was thalassemia followed by CKD and the general population with chronic liver disease (CLD) [**Table 5**]. The highest viremia was observed in the PWIDs group (70.70%) followed by the thalassemia group (65.20%).

HCV infection is one of the prevalent TTIs (Transfusion Transmitted Infections) among multi-transfused β-thalassaemic patients, leading to substantial health consequences in this high-risk population. A total of 250 anti-HCV positive samples were included in this group out of which 163 samples were RNA positive (65.20%). The most observable feature of this population is the age distribution. Most of the patients included in this study belong to the thalassemia group, aged under 20 years (~ 93%). Whereas, HCV infection is commonly reported at the age of around 40 years (348), in this study it was observed that thalassaemic patients in the age group 6-10 years were at more risk of HCV infection (**p-value:0.03**). This is due to these patients being compelled to go through multiple blood transfusions at this very early age (from age ~ 1 year). Moreover, due to inadequate screening of donor blood during the seronegative "window" period, serological tests such as Tri-Dot assays and ELISA, which are more widely employed for donor blood screening in blood banks, are ineffective in detecting active HCV infection. As a result, HCV-infected blood is being transfused to the thalassaemic patients. Thalassemia Children often face iron overload problems of the liver and in addition to HCV infection, it may lead to a severe case like hepatocellular carcinoma (349). It has also been found that male thalassaemic patients are more prone to HCV infection than females **(p-value:0.03)**. This disparity in HCV clearance rate based on gender is still an unresolved topic but there are a few potential causes. In most viral infections, baseline immune responses are stronger in females than in males, and low dosages of oestrogen have been proposed to influence antiviral innate and adaptive immune responses (350). It was also corroborated by previous studies that males are at higher risk of HCV infection (351). It has also been found that poor and remote thalassaemic patients were also at higher risk of HCV infection [**Table 6**]. This may be due to poor and remote people often not having much education and HCV-related awareness was also negligible in them. This also supports the previous study of HCV infection in the thalassaemic population (27,352). It is also reported that high serum ferritin level facilitates HCV replication

(353). This is a very common problem for thalassemia patients due to their repeated blood transfusions. It has been determined that thalassemia patients are at risk for developing liver fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) due to iron overload and HCV infection (199). The frequency of HCC in thalassemia patients was steadily rising, and the prevalence of cirrhosis varied from 10 to 20% in many studies from various populations of thalassaemic patients (United States, Italy, and Greece) (354–358). In this study, it was also found that HCV increases liver complications in thalassemia patients. HCV RNA-positive thalassaemic individuals showed higher liver enzyme profiles (Triglyceride, bilirubin, ALT, AST, and alkaline phosphates) than the HCV-negative thalassaemic patients [**Table 12**]. This study also showed that the thalassemia patients needed utmost care like proper NAT (Nucleic Acid Testing) based detection before blood transfusion, periodic check-ups with Anti-HCV ELISA and other govt. aided harm reduction protocols. Directly Acting Antivirals (DAA) are not recommended under 12 years of age, which can be another problem for thalassaemic children because most often they get infected under the age of 12 and remain untreated until the age of 12. But very recently, a study also showed that these HCV-infected thalassaemic children can benefit from a new recommendation of the DAA regime for them (359). In addition to treatments more accurate HCV detection methods are needed in blood banks to control HCV infections in thalassemia.

Another very common group, that is vulnerable to HCV infection in the Indian scenario is CKD or haemodialysis patients. There has been always a need to assess HCV epidemiology among CKD patients in developing countries (360). CKD patients often go through regular haemodialysis for their survival and they get infected with HCV from contaminated dialysis machines. This is due to a lack of stringent screening methods for HCV. Till now, anti-HCV screening has been performed to identify HCV infection. However, HCV infection in the window period cannot be detected just by ELISA and needs a more expensive PCR method.

This facility is very rare in dialysis centres and therefore dialysis machines get infected with HCV infection which in turn affects new CKD patients who have undergone dialysis from the same machine. In this study, a total of 155 anti-HCV positive CKD patients were enrolled, out of which 78 patients were found to be HCV-RNA positive. Therefore, HCV viremia among the CKD population is about 50.32%. This study also showed dialysis patients aged between 55 and 64 years were at higher risk of HCV infection (**p-value:0.01**). This is also corroborated by previous studies (361,362). It was also shown that the risk of HCV infection increases with the frequency of dialysis **(p-value:0.02)**. Individuals who receive dialysis more frequently (8–12 times per month) are more likely to have HCV infection. This is likely because patients were deliberately looking for various available dialysis facilities and were forced to receive dialysis for survival and get HCV infections through infected dialysis machines. It was also found that HCV prevalence was higher among those persons who had more advanced CKD (363). It was also found that patients residing in urban areas were more prone to HCV infection [**Table** 7]. It may be because of the more drastic lifestyle (like alcohol use, or smoking) of urban patients (364). HCV also increases the risk of ESRD (End Stage Renal Disease) development in CKD patients which ultimately needs kidney transplantation (363). It was also found that nearly about 76.15% (99 patients out of 130 patients) showed that they had several types of ESRD **(p-value: 0.04)** (**Table 10**). CKD patients with HCV who also have additional co-morbidities such as diabetes, hyperlipidaemia, cirrhosis, male gender, or age of more than 50 years, need extra attention (365). Patients with HCV infection may develop ESRD due to a variety of reasons. HCV may set off a series of immunological responses that target the kidneys and cause glomerulonephritis. Additionally, it was shown that dyslipidaemia and insulin resistance were linked to HCV (366) and thereby, increased the risk of renal disease. Clinical manifestations of kidney problems linked to HCV might include solitary proteinuria, nephrotic syndrome, or nephritic kidney disease, with or without impaired renal function. Membranoproliferative

glomerulonephritis (MPGN), membranous glomerulopathy (MG), focal segmental glomerulosclerosis, mesangial proliferative glomerular nephritis, or tubulointerstitial nephritis are the most common pathological abnormalities those are found in renal biopsy in HCV infected CKD patients (367). Additionally, MPGN was shown to be substantially more common in individuals with HCV-associated kidney illness than membranous glomerulopathy in a case-control study (0.36% vs. 0.05%; p-value < 0.0001). The most common involvement was type I MPGN linked to type II mixed cryoglobulinemia (368). Which is also reflected in this study. According to liver and renal clinical practice guidelines, HCV-infected patients must be checked for haematuria and proteinuria annually for early detection and prevention of glomerulopathies (369).

The third group that is very prone to HCV infections is the PWIDs group. 50% of new HCV infections in Europe were found to have a connection with PWIDs (370). Globally about 8.5% of all HCV infections occur in this population (227). In India near about 2 lac people belong to the PWIDs group and HCV prevalence among them was found to be almost (54.5-90 %) (371). Although India is sandwiched between the 'Golden Crescent' and 'Golden Triangle.' Yet, prevalence data of HCV among PWIDs are not very well established, however, it can be concluded from various studies that HCV sero-prevalence among PWIDs is moderate (30-50%) all over India, but there are also some pockets with a high seroprevalence of HCV among PWIDs (60-90%)(372). In this study, a total of 65 anti-HCV-positive PWIDs patients were registered, out of which 46 samples were found to be HCV RNA-positive. HCV viremia among PWIDs was found significantly higher (70.70%) than in any other group [**Table 9**]. There was no female found in the PWIDs population. This may be due to Indian females seldom consuming drugs and being conservative in comparison to men (373). It was also very difficult to include a female PWIDs candidate in this study due to socio-cultural barriers (374). Male IDU candidates were also very aggressive and they were also hiding their history of drug use.

Other high-risk groups like haemophilia and people with HIV were found to be very limited in this study.

HCV viremia in the general population with chronic liver disease was also estimated in this study. Although situated on the eastern Gangetic plain inhabited by 91 million people, there has been a paucity of information regarding HCV epidemiology among the CLD population in West Bengal. Overall, HCV RNA positivity was found in this group at about 58.73%. higher age bracket (52-67) people tend to have more risk for HCV infection. Blood transfusion is the main source of HCV infection in this population group (p-value:0.04). Blood transfusions can efficiently transmit the hepatitis C virus because they allow many infectious virions to circulate. This study found that blood transfusions, surgeries, and improper syringe and needle use are the main factors contributing to HCV transmission in the studied group. The most prevalent way that HCV is considered to spread in India is through blood transfusions (375). Blood transfusion during the window period may be the cause of this. Even with pre-transfusion blood screening, there may still be certain gaps that need to be closed, particularly about NAT-based screening, to stop HCV transmission (376). Since there is currently no vaccine, limiting transmission is a crucial strategy for stopping and managing HCV epidemics. Most of the decompensated patients in this study were found to have an active HCV infection (p-value: 0.02) [**Table 8**]. According to previous studies, HCV infection is responsible for 15–20% of all chronic liver disease (CLD) cases and nearly about 5–10% of all HCC cases in India (377). Direct-acting antiviral (DAA) treatments are very effective against HCV infection and show a high SVR (>98%) rate. The govt. of India launched NVHCP (National Viral Hepatitis Control Program) in 2018, so that, HCV-infected patients can get their free-of-cost medicines. Although the supply of these medicines was not very frequent(378). High-end NAT-based testing in blood banks and fully subsidized DAA treatments can help India get rid of HCV infection in future.

The second objective of this study was to analyse the genomic characterization of the isolated HCV strain in different high-risk group populations. There has always been very limited information about HCV genotype distributions in West Bengal. Varying HCV genotypes have varying efficacies in antiviral therapy (379). Thus, genotyping is critical for predicting the likelihood of response and determining the best duration of treatment (380). HCV genotype determination also plays a major role in the treatment of HCV when a patient fails to achieve SVR (HCV relapse cases)(24).

In the thalassemia population, six HCV subtypes were noticed and they were 1a (1.23%), 1b (12.27%), 3a (76.29%), 3b (8.59%), 3g (0.61%) and 3i (0.61%). HCV subtype 3a was the most prevalent one followed by subtype 3b and overall genotype 3 was the most predominant HCV genotype in the thalassemia population. HCV subtypes 3g and 3i were not previously reported from this region and this is the first time that these two HCV subtypes were found circulating in β-thalassemic patients of West Bengal [**Figure 14**].

In the CKD population, six HCV subtypes were identified which were as follows-1a (10.26%), 1b (3.85%), 1c (67.95%), 3a (6.40%), 3b (3.85%) and 4a (7.69%). HCV subtype 1c was more predominant in this group followed by1a. HCV subtype 1c is very rare and can only be found in Indonesia, subtype 4a was also not very common in this region. This indicates that future HCV evolution research is needed which will help in HCV control and management [**Figure 16**].

In the CLD population, seven HCV subtypes were found which were – 1a (5.06%), 1b (16.46%), 1c (2.53%), 3a (36.71%), 3b (34.18%), 3g (3.80%) and 3i (1.27%) [**Figure 18**].

In this study, a paradox in HCV genotype distribution was seen in this area. HCV subtype 3a was found to be more prevalent (76.69%) in this area among the thalassemia population [**Figure 14**]. Whereas, HCV subtype 1c was more predominant (67.69%) among the CKD

population [**Figure 16**]. On the contrary, the general population with CLD had HCV subtypes 3a and 3b almost in similar frequency [**Figure 18**]. Rare HCV subtypes 3g and 3i were found in the thalassemia population [**Figure 14**] and subtype 4a was found in the CKD population [**Figure 16**]. For a clear understanding, **Figure 20** may be followed.

However, the exact reason for this paradox is unknown. It can be postulated that HCV genotypes evolve due to the host immune factors and it was also proved that HCV genotype changes with race, geography, sex, age, and other factors (18) and that can be a probable explanation for this. To adequately address this, however, further in-depth investigations at both the host and virus levels are required. Another interesting observation is the higher predominance of HCV subtype 3a among β-thalassaemic patients. It has previously been shown that individuals with β-thalassaemic disease in Iran have a similar pattern of HCV genotype 3 (subtype 3a) prevalence (381). The "Global thalassemia belt" (382) of central, middle, and Southeast Asian nations is the primary distribution area for HCV subtype 3a (383). Thalassemia instances on the South American subcontinent were also found in Brazil (384), where, interestingly, there was also a high prevalence of HCV genotype 3a (385). Nevertheless, more studies are needed to confirm it.

Phylogeographical studies revealed that subtype 3a resembled strains from neighbouring countries like Sri Lanka, Russia, Pakistan, Myanmar, Iran, and Thailand [**Figure 21**]. Subtype 1c showed relatedness with isolates from Indonesia, China, Cameroon, and Myanmar [**Figure 22]**. HCV subtype 3b isolates found in this region shared relatedness with China, Myanmar, Vietnam, Thailand, and other Southeast Asian isolates [**Figure 23**]. Subtype 1b was found to share common ancestors with Japan, Myanmar, and Thailand isolates [**Figure 24**]. Subtype 1a shared relations with various countries' isolates like China, Venezuela, Germany, and Iran [**Figure 25**]. Subtype 4a might be migrated from Saudi Arabia **[Figure 26**].

Overall, seven HCV subtypes were identified from this region among which 1c, 3g, 3i, and 4a are very rare. So, many HCV subtypes congested in India can give rise to new HCV subtypes which can be a future problem. HCV Genotype 3 is found to be the most prevalent in this area which is also associated with accelerated fibrosis, steatosis, and HCC (386,387).

Direct-acting antivirals (DAA) have greatly improved HCV treatment options in recent years. Side effects of HCV DAAs are also negligible (378,388). These DAAs are more effective than the previous treatment option (PEG IFN+Ribavirin) (389) and the SVR achievement rate was also found to be very high (388). In India, DAA-based treatment guidelines were established in the year of 2018 under the NVHCP program. Under this program, patients get free treatment for HCV. Before this initiative, this treatment option was out of reach for middle- and lower-income families. Despite this initiative, DAA-resistant mutations have come into play (63,174,254,255,259,260,262,280,390–392) and can be a great concern for DAAs-based therapies in future. Therefore, naturally occurring DAAs-RAS (Resistance Associated Substitutions), mutations which confer drug resistance, carry immense importance in future HCV control planning. Other than that, HCV mutations also play a major role in the pathogenesis and progression of liver disease (393). Altogether, it can be said that a details analysis of HCV proteome can open a new path towards drug development and HCV control(394). Therefore, the third objective of this study was set to Mutational analysis of HCV non-structural gene 3 (NS3) and to find out the important mutations that are associated with chronic HCV and prediction of treatment outcome. In this study, the HCV-GLUE tool (http://hcv-glue.cvr.gla.ac.uk/#/home, last accessed on 02.04.2024) was utilised to identify naturally occurring resistance NS3 mutations towards NS3 inhibitors. A high degree (in about 97% population) of category II mutation was observed in this area. A novel NS3 mutation N224T was identified in this study which may be involved in decompensated chronic liver disease (DCLD). This N224T mutation was found to be present in 70% cases of DCLD cases

[**Figure 29**]. **Y56Y+Q168Q+I/V170I** and **A166S** mutation was found in this area which confer drug resistance towards Glecaprevir, Paritaprevir, and Grazoprevir [**Figure 30**].

As mentioned earlier, the development of HCV with resistance-associated substitutions caused by mutations in the HCV genome is a major concern in DAA treatment. Moreover, pregnant women still cannot be treated with these DAAs (395). DAAs are also involved with DDI (drug-drug interaction) due to which patients with other co-morbidities cannot be treated with DAAs(396). The cost of DAAs against HCV is also very high. Altogether these problems indicating a vaccine for HCV is very much needed. But high error rate of HCV RdRp is the primary barrier to HCV vaccine development. The high error rate of HCV RdRp causes random mutations by which HCV can bypass T-Cells. Therefore, the development of an antibody against a particular HCV subtype cannot give rise to enough protection against other subtypes. To address this problem novel epitope-based vaccine design approach can be utilised. Immunoinformatics combined with a multi-epitope vaccine development method has recently demonstrated encouraging outcomes in several vaccine development and manufacturing procedures(397–399). This technique has the advantage of having a smaller primary immunogen (smaller peptides) and producing stronger immunogenic responses (400). This property may be advantageous in the event of immunogens with considerable variability since epitopes from various subtypes can be included in a single vaccine due to their smaller size. Recently, the epitope-based technique has demonstrated better outcomes than the traditional way(400–402). Besides, both NS3 protease and helicase are proven to be very critical for viral replication and life cycle, and they are also considered one of the finest targets for the development of anti-HCV therapy techniques (72,282,403). The HCV NS3 protein is a prominent target for vaccine development against HCV(69,282). NS3-specific T-cell responses are also linked to viral clearance (74,283). It has also been found that the NS3 protein of HCV can trigger T-cell responses better than other proteins (284,404,405). Therefore, the fourth

objective of this study was to find NS3-host-specific T-cell immune epitopes and their characterization. HCV genotype 3a is the most prevalent one in this region therefore, in this study, NS3-based epitopes of HCV 3a were predicted using an in-silico method. Epitopes were also tested using the main four characteristics of antigenicity, allergenicity, toxicity, and human homology. Novel prediction approaches for epitope screening include IFN-γ (MHC-I and II) and IL-4 and IL-10 (MHC-II). Although, prior research predicted HCV epitopes using an in-silico technique (327,406–408). However, they either did not target the same HCV protein or there were not enough filtering epitopes with distinct filters. The contemporary method states that when selecting epitopes, they should be non-toxic, non-allergenic, and antigenic (409). It is important to closely examine the human homology of epitopes since HCV epitopes that are identical to human proteins can result in immunological suppression and lead to chronicity (410). Because IFN-γ is crucial for HCV clearance, IFN-γ prediction is also a significant prediction parameter for epitope predictions (411). This study also considered filtering factors based on IL-4 and IL-10 to predict MHC-II epitopes. which are also needed to anticipate MHC-II epitopes (412). A total of 119 MHC-I epitopes and 436 MHC-II epitopes were predicted which were finally reduced to 37 MHC-I and 16 MHC-II epitopes after application of all filtering parameters [**Figure 40** and **Figure 41**].

It is very important to analyse the conservancy of these epitopes and validate them *in-silico* and *In-vitro* methods (401). Therefore, the last objective was set to Conservancy analysis and validation of these epitopes. The epitopes of MHC-I and MHC-II were also examined for conservation analysis. More than 80% of conserved epitopes were taken into consideration for further docking studies using various MHC-I and II alleles. After conservancy analysis 11 MHC-I epitopes and 6 MHC-II epitopes were found to match that criterion (>80% conservancy) [**Table 14** and **Table 15**]. Subsequently, highly conserved epitopes were examined for molecular docking and optimization. Twenty-three distinct MHC-I allele types,

which are common in the Asian population, were docked with each MHC-I epitope, and twelve distinct MHC-II sub-chains, which are also common in the Asian population, were docked with each MHC-II epitope. Conventional approaches for discovering and screening T-cell epitopes with multiple parameters (such as antigenicity, allergenicity, toxicity, etc.) are challenging tasks, so, *In-silico* screening and conservation analysis of T-cell epitopes before undertaking *In-vitro* or *in-vivo* research can be more economical and time-saving (322,413–416).

The predicted MHC-I epitopes have good RMSD values, demonstrating their ability to bind a variety of alleles [**Figure 42**]. Nevertheless, compared to MHC-I alleles, MHC-II alleles were shown to be more selective for epitopes [**Figure 43**]. It is also crucial to remember that every MHC-II molecule has two distinct chains and a flexible antigen binding groove; also, to trigger a potent reaction, the MHC-II molecule requires super dimer structure (417,418). After *in-silico* docking analysis, the top 11 (MHC-I and MHC-II) epitopes were selected by RMSD scoring and validated with *In-vitro* validation. Peptides were synthesised using the f-MOC chemistry method and purified using HPLC. All 11 epitopes showed good T-cell response in the PBMC T-cell proliferation assay [**Figure 45** and **Figure 46**]These epitopes were also found to induce the IFN-γ signalling pathway [**Figure 47** and **Figure 48**]. It is essential to confirm *in-silico* predicted epitopes through *In-vitro* testing before employing them as a vaccination candidate (401). All predicted epitopes cannot induce T-cell responses, which are vital for immunotherapy and vaccine development (419).

Some epitopes were identified to be located on active sites of the NS3 protease helicase enzyme complex (279). Epitope $_{3}$ITAYAQQTR$_{11}$, $_{454}$DAVSRSQRR$_{462}$, $_{492}$CECYDAGCSW$_{501}$, $_{432}$VEQYVDFSL$_{440}$, and $_{548}$SQTKQQGLNF$_{557}$ among the MHC-I epitopes were found to be located on the active site of the enzyme complex. Other epitopes are also located near active sites. The amino acids 551K and 552Q are found in both the active site area of the NS3 helicase

and MHC-II epitopes. Epitopes located on active site regions can be advantageous since active sites are often essential for enzymatic activity during viral replication and transcription (420).

In recent years, the CHAD3-NSmut/MVA-NSmut T-cell vaccine (421), which is a combination of chimpanzee adenovirus and modified vaccinia Ankara (ChAD3 and MVA) viral vector encoding non-structural genes (NS3-NS5 regions) with mutated NS5B form HCV 1b subtype, was not able to prevent chronic infections in the PWIDs cohort (clinical trial NCT01436357)(310) in a very promising way. Although the precise cause of this result is yet unknown, it is possible that PWIDs' immune-suppressive characteristics are to blame. Numerous studies showed that the PWIDs population has lower levels of NK cell activity, phagocytosis, and antigen-dependent cytotoxic (ADCC) killing. Phagocytosis is a crucial process for antigen presentation (422), and natural killer cells (NK cells) are essential for the clearance of HCV (423,424). The failure of this vaccination may be due to both impairments in the PWIDs group with poor ADCC. The great degree of HCV viral diversity might be one more reason for this vaccination failure. Research has shown that changes in the HCV virus impact the processing of epitopes (425). It has also been observed that immunological escape is caused by epitope alterations (426–428). Nevertheless, HCV genotypes 3 and 1a account for the majority of PWIDs patients who have been infected with the virus. In contrast, genotype 1b infection is uncommon in PWIDs cases (429). Conversely, non-structural areas from HCV subtype 1b contribute to CHAD3-NSmut/MVA-NSmut (421). Since HCV genotype 3a or 1 is more common in the PWIDs community, T-cells with memories of HCV 1b will probably not be highly efficient. On the other hand, compared to CHAD3-NSmut-1b, a recent epitope-based study also demonstrated a superior immune response in mice (311). In this investigation, conserved epitopes from different HCV genotypes were considered, and even those that CHAD3-NSmut-1b did not show higher positive reactions. To prevent HCV and to identify and validate immunogenic HCV epitopes, an alternative strategy based on epitopes might be

quite helpful. In the development of HCV vaccines, the NS3 region of various subtypes is very crucial.

Other than these objectives the study also focuses on how these epitopes conserve in other HCV subtypes which are prevalent in the Indian scenario (Section 7.7). According to this study and some previous studies (Biswas et al., 2017; K. Saha, Firdaus, Biswas, Mukherjee, Sarkar, Chakrabarti, Sadhukhan, et al., 2014) HCV subtype 1a, 1b, 1c, 3a, 3b, 4a, 6h, 6k, and 6n were found in Eastern and Northeastern India. Mutational analysis found that among predicted epitopes at least one epitope is found to be conserved in all HCV subtypes [**Figure 56**].

It was also reported that a single epitope can also confer protection (430–432). Therefore, it can be said that these epitopes can also be effective for pan-genotypic vaccine construct. However, Allelic variation (MHC polymorphism) must be taken into consideration for epitope analysis and prediction (433). Nevertheless, Global population coverage for super types A_01_01, A_02_01, A_03_01, A_24_02, B_07_02, and B_44_03 was 90% (434). Which is also included in this study. This suggests that the epitopes identified in this study could be helpful not just in Asian nations but also in other regions of the world. Considering all, the allelic predominance of an area and molecular epidemiology can provide an approximate estimate of the specificity of epitopes.

Upon filtering and conservancy analysis, all MHC-I and II epitopes were chosen in advance of peptide synthesis. Only the optimal epitopes identified by docking analysis were used in the *In-vitro* experiment. *Ex-vivo* investigations did not corroborate the variance observed in other subgroups. Further study using molecular dynamics modelling may be needed to determine the functional and structural significance of altered epitopes across all subtypes (435). These points can be addressed in future.

7 Future prospects:

Epidemiology, gene sequencing and Immunoinformatics can altogether be employed to efficiently predict epitopes from full HCV proteomes of all subtypes and then linked together with linkers can yield a hypothetical protein which can further clone into *E. coli* to produce HCV vaccine in future [**Figure 57**]. Other than this these epitopes can also be used as prophylactic candidates.

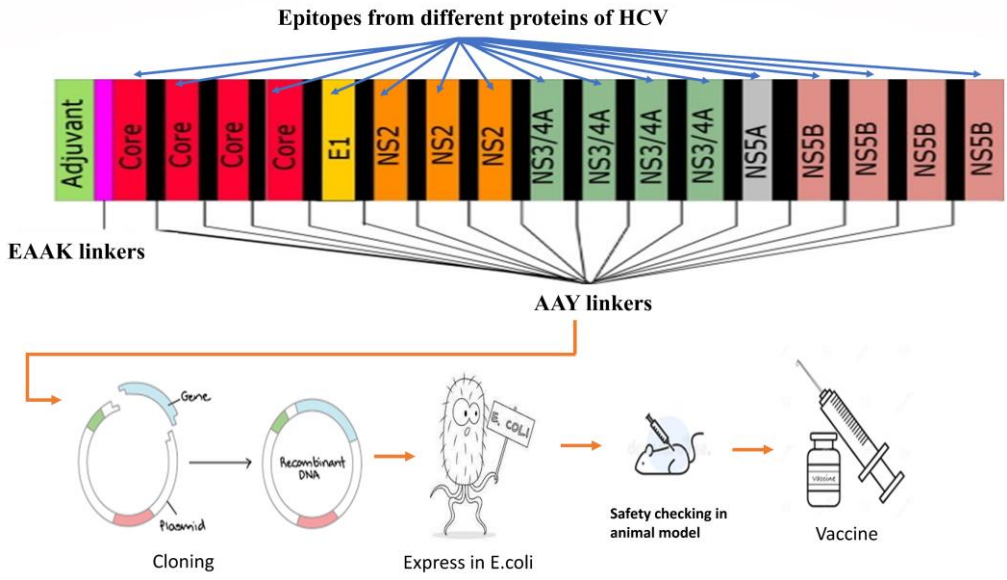


Figure 57: Plan for future HCV vaccine.

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9 Publications:

1. **Dutta S,** Nath S, Ahammed M, Das R, Bakshi S, Majumder M, Ghosh A Verma P, Baskey U, Dutta S, Sadhukhan PC. Assessment of direct-acting antiviral therapy in different HCV genotype-infected chronic liver disease patients of West Bengal, India. Indian Journal of Medical Research (IJMR) [Accepted for publication].

2. **Dutta, S**.; Biswas, A.; Bakshi, S.; Choudhury, P.; Das, R.; Nath, S.; Chowdhury, P.; Bhattacharyya, M.; Chakraborty, S.; Dutta, S.; et al. Molecular Epidemiology of HCV Infection among Multi-Transfused β-Thalassemia Patients in Eastern India: A Six-Year Observation. *Thalass. Rep.* **2023**, *13*, 165-178. https://doi.org/10.3390/thalassrep13030016

3. Samadder RK, Ray G, **Dutta S**, Hazra A, Sadhukhan P, Chowdhury A, Ray R, Ahammed SM. The Efficacy and Safety of Sofosbuvir and Daclatasvir Treatment in Children and Adolescents With Thalassemia and Hepatitis C Virus Infection. J Clin Exp Hepatol. 2024 May-Jun;14(3):101310. doi: 10.1016/j.jceh.2023.101310. Epub 2023 Dec 1. PMID: 38264577; PMCID: PMC10801307.

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10 Posters and abstracts:

1. Finding suitable Hepatitis C virus NS3-based T cell epitopes using a combination of in-silico and in-vitro approaches.
   Supradip Dutta, Amlanjyoti Dhar, Moumita Majumdar, Sagnik Bakshi, Raina Das, Shreyasi Nath, Anwesha Ghosh, Shanta Dutta, Provash Chandra Sadhukhan.
   [Accepted for presentation in upcoming **HCV-Flavi 2024**, Oxford, UK]

2. Genomic Diversity of HCV among High-risk group population in Darjeeling.
   Supradip Dutta, Raina Das, Sagnik Bakshi, Shreyasi Nath, Dr. Provash Chandra Sadhukhan (**16th ASCODD,2022, Kolkata**) [poster]

3. Genomic diversity of hepatitis C virus among high-risk group population in the Eastern part of India
   Supradip Dutta, Sagnik Bakshi, Upasana Baskey, Priya Verma, Aritra Biswas, Raina Das, Promisree Choudhury, Shanta Dutta and Provash Chandra Sadhukhan. (**Molecular Virology Meeting, 2019, IIT-Kharagpur**) [Poster]

4. Comparative study on genomic diversity of Hepatitis C virus among people who inject drugs (PWIDs) in Eastern and North-Eastern states of India
   Supradip Dutta, Aritra Biswas, Upasana Baskey, Priya Kumari Verma and Provash Chandra Sadhukhan. (Society of Tropical Medicine, 2018, Kolkata) [Poster]

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