Prevalence of HBV and Associated Genetic Polymorphism in Chronic HBV Patients of KPK-Region in Pakistan

By

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DEDICATED

TO

My parents and family

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List of Abbreviations

ALT	Alanine aminotransferase
AMH	Anatomically modern human
APRI	Aspartate aminotransferase to platelets ratio index
AST	Aspartate aminotransferase
CD4T	Complementarity determining 4 T cell
CD8T	Complementarity determining 8 T cell
CDC	Center for disease control
CDK2	Cyclin-dependent kinase 2
Cdna	Complementary DNA
CH	Cholesterol
CHB	Chronic hepatitis B
CHC	Chronic hepatitis C
CI	Confidence Interval
CJP	Contact with jaundice patient
CTD	C-terminal domain
CTL	Cytotoxic T lymphocyte
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
EIA	Enzyme immunoassay
ELISA	Enzyme linked immunosorbent assay
EM	Electron microscopy
ER	Endoplasmic reticulum
ESCRT	Endosomal sorting complex required for transport

FDA	Food and drug administration
GWAS	Genome wide association studies
HBcIgG	Hepatitis B core immunoglobulin G
HBcIgM	Hepatitis B core immunoglobulin M
HBeAB	Hepatitis B envelope antibody
HBeAg	Hepatitis B envelope antigen
HBsAb	Hepatitis B surface antibody
HbsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HDV	Hepatitis delta virus
HIV	Human immunodeficiency virus
HMC	Hayatabad Medical Complex
HSPG	Heparan sulfate proteoglycans
IFN/RBV	Interferon/ribavirin
IFNL3	Interferon lambda 3
IFN-ϒ	Interferon gamma
IL-2	Interlukin-2
IU	International unit
KPK	Khyber Pakhtunkhwa
LC	Liver cirrhosis
MAF	Minor allele frequency
MBOAT7	Membrane bound O-acyltransferase domain-containing protein 7
MSM	Men who have sex with men
NAFLD	Non-alcoholic fatty liver disease

NASH	Non-alcoholic steatohepatitis
NCBI	National center for biotechnology information
NF-Kb	Nuclear factor kappa b
NK	Natural killer cells
NKT	Natural killer t cells
NLR	(NOD)-like receptors
NLS	Nuclear localization signals (sequence)
NTCP	Sodium taurocholate co-transporting polypeptide
OBI	Occult hepatitis B infection
OPD	Outpatients department
OR	Odds ratio
ORF	Open reading frame
PAMP	Pathogen associated molecular patterns
PCR	Polymerase chain reaction
Peg-IFN	Pegylated-interferon
PI	Protease inhibitors
PKR	Protein kinase receptor
PNPLA3	Patatin-like phospholipase domain containing 3
QAU	Quaid-i-Azam University
RT-PCR	Reverse transcription-polymerase chain reaction
SD	Standard deviation
SGPT	Serum glutamate-pyruvate transaminase
SNP	Single nucleotide polymorphism
SOD	Superoxide dismutase
SOP	Standard operating procedure

SR	Spontaneously recovered
SRPK	Serine-rich protein kinase
ssRNA	Single stranded ribonucleic acid
STAT4	Signal transducer and activator of transcription 4
TB	Total bilirubin
TBE	tris/borate/EDTA
TFA	Tubule forming agent
TG	Triglyceride
TGF	Transforming growth factor
TLR	Toll-like receptors
TM6SF2	Transmembrane 6 superfamily 2
TP	Terminal protein
UV	Ultra-violet
WHO	World health organization

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Summary

Hepatitis B virus (HBV) infection is a global public health concern that affects more than 2 billion people throughout the world. Among these 400 million develop chronic hepatitis infection. In year 2015 alone, 257 million individuals were living with chronic HBV infection (WHO data) while 887000 fatalities were reported due to HBV hepatocellular carcinoma and cirrhosis. Although, chronic hepatitis B (CHB) infection is rare in developed countries, in Pakistan HBV morbidity and mortality is high. One important aspect of this higher morbidity and mortality is host genetic factors. This study consists of two parts; First, 946 blood samples were screened for the prevalence of HBV and co-infection of other hepatitis viruses (HDV & HCV) in general population. Overall percentage of HBsAg-positive patients remained 22.41% (prevalence: 224.10/1 000; CI: 0.197 5 ± 0.250 7) with the highest incidence rates among relatively younger age groups (20–29 years). The prevalence of HBV–HDV co-infection was found to be 46.75/1000; CI: 0.0318 ± 0.062, while HBV-HCV co-infection was found 7.55% (16/212). In HBsAg-positive patients, anti-HBc-total was detected in 86.79%, while 25% were positive for anti-HBc-immunoglobulin M. among these patients, HBV DNA was detected in 64.13% and 10.85% were co-infected with HDV. Different symptoms were associated with the prevailing infection, including malaise (62%), anorexia (66%) and fatigue (73%). The most commonly associated symptom was abdominal discomfort. Among these patients, certain risk factors, including surgery, visit to dentist and intravenous infusions were frequently associated with the infection ($\chi 2 = 95.23$; df = 11; $P < 0.000\ 1$).

In the second part of this study, 354 blood samples (240) HBV infection, 57 clearance and 57 healthy controls) samples were collected. Based on different clinical parameters patients were categorized into healthy, spontaneously recovered, chronic with steatosis and without steatosis groups. Among 240 chronic HBV patients, 44 (18%) were found to have steatosis and n=196 (82%) had no steatosis. The gender-based distribution for the studied participants revealed that 70% were males and 30% females. Although the median age of the recruited patients was 29 years. Some common signs and symptoms among studied patients including abdominal discomfort, anorexia, fatigue, and malaise were witnessed.

Demographic analysis exhibited that most of the patients in this study belonged to Peshawar, Khyber Agency and Charsadda, while 52% of the patients were from rural areas and 48% from urban centers of the country. Similarly, age group of 20-29 was predominant. While distribution of risk factors showed that the most common risk factors included injections, IV infusions, and contact with jaundice person. Similarly, educational background of the studied individuals depicted that individuals with primary education were in the highest percentage that was followed by intermediate and matric levels of education. Occupational data of the studied group showed that most of the female patients were housewives followed by students and laborer. HBV DNA was observed in 64% of the patients, while the rest were PCR negative. The average viral load in log 10 in both male and female patients was reported to be 5.34 IU/ml and 5.21IU/ml respectively. Classification of the studied patients showed that all were broadly categorized into three groups that included clearance, chronic HBV and steatosis group.

After genotyping of SNPs association were analyzed in healthy individuals, cleared, chronic HBV and steatosis cohorts. Significant differences between baseline characteristic of cleared and HBV positive were noticed particularly in terms ALT, AST and platelets. While in the clinical attributes, steatosis and non-steatosis group were significantly different in terms of age, ALT, AST, cholesterol TG and platelets levels. The *STAT4*rs7574865TT genotype, observed in 6% of the patients, was associated with chronic persistence (p=0.048). In contrast rs12979860 TT genotype, witnessed in 5% of patients, was not significantly different between both cohorts. We compared these genotypes among male, female, steatosis and non-steatosis groups however no significant association was found. Further, we analyzed the above mentioned genotype through multiple logistic regression analysis. We found that *STAT4* rs7574865 is varied in dominant and adjusted model (adjusted with age and gender) of chronic and recovered cohort while remained insignificant in steatosis cohort. On the other hand, for *IFNL3* rs12979860, no significant association was observed in chronic and steatosis cohorts (multiple logistic regressions analysis). The *PNPLA3* rs738409 GG genotype, observed in 3% of the patients, was linked with persistence of HBV infection (p=0.031) and hepatic steatosis (p=0.033). Similarly, transmembrane 6 superfamily member 2 (*TM6SF2)* rs58542926 TT genotype, detected in 4.6% of the patients, minor (TT) allele

of *TM6SF2* rs58542926 was significantly linked with patients having steatosis when compared to those without steatosis (p=0.024).

The*MBOAT7* rs641738CC genotype was witnessed in 25% of the patients and was not significantly diverse between chronic, healthy and steatosis cohorts. We also analysed the above mentioned genotypes among male and female but no significant association was noticed.We analyzed *PNPLA3* rs738409 *TM6SF2* rs58542926 and *MBOAT* rs641738 genotype through multiple logistic regression analysis. We observed that *PNPLA3* rs738409 was different in dominant and adjusted model (adjusted with age and gender) of chronic and healthy, while stayed significant in steatosis cohort also. On the other hand *MBOAT7* rs641738 had showed no significant difference in chronic and steatosis cohort after analyzing by multiple logistic regressions. Baseline clinical variables among HBV infected patients, respective to their *IFNL3* rs12979860 genotype were also examined. Our results demonstrated subjects with CT genotype of *IFNL3* rs12979860 have significantly lower levels of cholesterol when compared with those with CC and TT genotype, while didn't notice any significant link among rs12979860 genotype (CC versus CT versus TT) with any of the other clinical variables mentioned above.

We further explored, different clinical variables among HBV infected patients respective of their *STAT4* rs7574865 genotype, which exhibited no convincing evidence of significant association among rs7574865 genotype, comparing minor genotype to other genotypes (GG versus GT versus TT) and any other clinical variables like (age, ALT, AST gender, HBV-DNA cholesterol, TG, platelets and total bilirubin). Next, we examined the clinical variables association with each genotype. We detected that subjects with rs738409 CG genotype have significantly lower ages levels compared to those with CC and GG genotype (p=0.009), we also observed that the subject with rs738409 TT genotype have higher ALT level when compared to CC and CT genotype (p=0.027).

Next our results revealed that subjects with rs641738 CC genotype have significantly higher cholesterol level when compared to CT and TT genotype (p=0.014). While no evidence of significant association observed between *TM6SF2* rs58542926,

*PNPLA3*rs738409, or *MBOAT7* rs641738 genotype with any of the other clinical variables (i.e., gender, AST, HBV-DNA, total bilirubin, Platelets and TG. Moreover patients with relatively high serum HBV DNA level have significantly high serum cholesterol (p=0.044) and triglyceride (p=0.010) in comparison to low HBV DNA level. While none of the other variables was associated with HBV-DNA levels. In conclusion, it is the first report that highlighted HBV/HDV infection in OPD patients in KPK region, which indicates higher prevalence rate of HBV/HDV infection among patients which have no prior history of any kind of hepatitis and majority of these patients belonged to the younger age group. In addition, serological and viral DNA based evidence suggest active prevalence of HBV infection among majority of the patients which indicates higher risk of further HBV transmission by these patients. Significant efforts are required in order to control the co-infection of HBV/HDV in the population of KPK in Pakistan. Likewise, the second part of this study establishes that STAT4 but not IFNL3 polymorphisms are associated with HBV infection persistence in populace of KPK region in Pakistan. It further reflects the association of polymorphisms in PNPLA3 and TM6SF2 with hepatic steatosis and HBV viral infection persistence in Pakistani population. In addition, the same analysis concludes that polymorphism in the MBOAT7 gene is not related to steatosis or persistence of HBV infection in Pakistani population. Although further detail studies will be required to clarify the underlying molecular and cellular mechanisms of this genetic association and the differential immune response between the two common form of infective viral hepatitis C and B.

Introduction

HBV is a major public health concern that causes inflammation of hepatocytes in the liver. HBV infection is potentially life-threatening and a leading cause of both chronic and acute liver disease globally. Infection with HBV results into loss of millions of precious lives worldwide. The persistence of the disease in humans is attributed to the complex life cycle of the virus and its ability to use exclusive few protein products in a multifunctional way to maintain and escape detection by immune system and elimination. HBV is enveloped DNA virus however; its DNA is not completely double stranded. Genome of HBV is around 3.2 kb in length, organized into four open reading frames. On the basis of its nucleotide sequences, HBV is classified into almost eight different genotypes. The genotypes of HBV are denoted from A to H and geographical distribution of each genotype varies.

HBV is highly infectious and can easily transmit from one person to other, via contaminated body fluids. Approximately 500 million individuals throughout the world are suffering with chronic hepatitis B or C and more than 250 million acquired chronic HBV infection (WHO 2020). Moreover, the World Health Organization (WHO) has estimated HBV associated death rate around 1.34 million per year mainly due to liver cirrhosis or liver scaring and hepatocellular carcinoma (HCC). Similarly about 66% of the world population is residing in regions facing higher frequencies of HBV infection. According to the WHO standards, countries having infection rates above 5% of their general population are considered highly endemic. Some of the African countries have already been declared endemic. Prevalence rate of HBV is significantly higher in several South East-Asian countries. In Asia and Western Pacific about 75% of the individuals are chronic carriers of HBV (Gust 1996). Around 3-9% population of Pakistan is infected with HBV (Lee 1997). Now it is widely accepted that HBV/HDV co-infection leads to more severe liver disease, cirrhosis development, higher liver decompensation and increase liver-related death risk, when compared with HBV infection alone. Studies showed almost 5% of the HBV carriers are co-infected with Hepatitis D virus (HDV) which result into almost 15 million individuals around the world with HDV infection (Jalil *et al.*2016).

Introduction

Pakistan falls in highly endemic regions of HBV infection (Noorali *et al.* 2008) and at least 9 million individuals are infected with hepatitis B (Hakim *et al.* 2008). The reason for elevated number of positive cases may be the lack of awareness, improper facilities of health and economically poor background status (Alam *et al.* 2007). About 80% cases of acute hepatitis B infection get cleared within 6 months. HBV infection may become chronic in 20% of the cases which may develop into chronic liver infection. The possible risk of infection in newborn is more than 90% when mother is HBe-Ag positive. After 6 months the infection risk declines from 90% to 25% at age of five years and it further declines to 10% at fifteen years of age. In adults the possible risk of infection is different (2%-5%) (WHO 2004).

Globally around 8 to 16 million HBV infections are reported each year, attributed to insecure and excessive use of injections (Kane *et al.*1999). In Pakistan, among general population large scale studies have not yet been conducted on prevalence of HBV, (multiple studies were conducted on stratified groups) therefore to assess true prevalence in general population and incidence rate of HBV infections are not easy to calculate. However, several independent reports described the prevalence of HBsAg among different population strata in Pakistan including blood donors (6.0%), children (2.5%), pregnant women (2.6%), high risk groups (12.3%), healthcare workers (13%), army recruits (2.4%), and patients with provisional diagnosis of hepatitis. Most of the investigations have been focused on stratified groups like patients with hepatitis, individuals at higher risk of infection and blood donors. The prevalence ratio of HBV infection in internally displaced peoples (IDPs) of Malakand division during the war against terrorism was observed up to 21%, among which 21.5% were female and 78.5% males. Major HBV positive population belongs to Dir (lower) and Malakand district. The carriers with high risk of HBV infection were depicted in elders (46-60 years) i.e. 29.13%, whereas the children (less than 15 years) were found with lower prevalence (Khan *et al.* 2011). Very limited data is present on HBV infection which does not portray vibrant picture of frequency rate of HBV in all over the country and specifically in KPK region. Majority of studies have targeted only a small group of individuals therefore they don't show precisely the general infection prevalence rate in Pakistan (Hakim *et al.*2008).

Another prevalence study was conducted by screening healthy individuals, before their orientation in armed force which indicated HBs-Ag in the range of 3.2% (Mirza *et al.* 2006). Drug users, patients getting blood products and health care personnel are at higher risk and the observed prevalence of HBV in this group ranged from 2.4-20%. The risk factors associated with blood transfusion, therapeutic injections, dental extraction and surgery were also reported (Bosan *et al.* 2010; Khan *et al.*2008). While some studies and data demonstrated that patients subjected to provisional diagnosis showed 10-45% prevalence of HBs-Ag.

HBV infected individuals' show four important phases including the first one which is immune tolerant phase, where patient's ALT level is usually normal with higher HBV DNA (> 20,000 IU/mL) and they are HBe-Ag positive. The second one is immune clearance phase in which ALT level of patient is not normal, with HBe-Ag positive and higher viral DNA quantities (overall higher but lower than immune tolerant phase). The third phase is known as inactive phase in which individuals are negative for HBe-Ag, with normal ALT level and low viral load. The fourth and the final phase is the immune escape phase in which patient's level of ALT is abnormal, either low or higher load of viral DNA but is negative for HBe-Ag. However, these four phases are not in serial order in all chronic infected individuals, any patients can progress to any phase during infection (Brooks *et al.* 2013). During seroconversion of HBe-Ag, decline in the vDNA levels and reduction in liver inflammation was witnessed (Chen *et al.* 2002). The viral DNA load is an important parameter for the diagnosis of HBV (Chen *et al.* 2011).

HBV mainly targets hepatocytes, whereas chronic HBV infection is the worldwide cause for the development of HCC (Bouchard and Navas-Martin 2011; Neuveut *et al.* 2010). Although there is a solid link between HCC and chronic HBV infection, however mechanisms of HCC development remain absecure. Development of HCC involves a complex sequence of destruction of HBV-infected hepatocytes by immune system and accompanied by regeneration of hepatocyte, involvement of some proteins of HBV like HBx, and integration of viral genome inside the host genome (Bouchard and Navas-Martin 2011; Neuveut *et al.* 2010).

Despite treatments are available for the chronic HBV infection, the emergence of mutants may lead to resistance to anti-HBV drugs, which poses an obstacle to continue the nucleoside analogue treatment. Furthermore, antiviral treatments currently available can restrict the HBV but cannot completely remove the targeted virons. This is because of HBV nuclear-localized cccDNA, that remains a hurdle for the treatment of these infections (Cheng *et al.* 2011; Yang *et al.* 2015). Although there is considerable advancement in understanding mechanism of HBV infection, replication and clearance, understanding HBV lifecycle needs further investigations.

HBV shows remarkable genetic variation i.e. $1.4 - 3.2 \times 10^{-5}$ nucleotide substitution per site/year (Orito *et al.* 1989). Up till now at least eight HBV genotypes have been recognized by various studies, designated as A-H, with nucleotide sequence difference s >8%, but <17% among each genotype (Seeger *et al.* 2014; Locarnini *et al.* 2013). Furthermore, two new genotypes were also described. Genotype I has a genetic variability of about 8% and has a robust similarity with genotype C, which make its classification of distinct genotypes more controversial than the other well-known and accepted genotypes (Huy *et al.* 2008 and Kurbanoy *et al.* 2008). Due to genetic recombination of C genotype and HBV gibbon, a novel 10th genotype i.e. genotype J, has recently been described (Tatematsu *et al.* 2009). The genotype D is the most dominant genotype existing in population of Pakistan (Masood *et al.* 2019).

Acute HBV infection has a wide range of clinical features resulting in asymptomatic infection, however rarely develops into life-threatening fulminant hepatitis, which needs rapid transplantation. Majority of the chronic HBV infections are manifested by subclinical disease and along with prognosis that results in development of HBV-related hepatocellular carcinoma (HCC) and liver cirrhosis. The latent infection is directly related to increased risk of mortality (Shen *et al.* 2015).

Effective vaccination strategies are established to prevent infection with HBV virus, liver cancer and chronic liver infections. Currently different antiviral drugs are used for the treatment of HBV chronic infections including oral agents or pegylated interferon-alpha, adefovir, lamivudine, tenofovir and telbivudine.

Worldwide seroconversion of HBs-Ag, occurs rarely in chronic HBV patients. (Masrour *et al.* 2020) HBV acute and chronic infections are distinguished by serological markers. In acute phase serum of HBV infected individual would be positive for HBs-Ag, IgM antibodies produced against the viral core (HBc-Ab IgM) and while antibodies to HBs-Ag would be absent. However, chronic patients should be positive for HBs-Ag (after 6 months) negative HBs-Ab and positive IgG against the viral core (HBc-Ab IgG) (Trepo *et al.* 2014). A spontaneously recovered patient should have positive HBs-Ab along with IgG HBc-Ag without the presence of viral DNA. The risk of conversion of acute hepatitis B to chronic infection is dependent on age. Risk of chronic infection is relatively higher (about 95% individuals) in infants, when compared to the adults that is comparatively low (>5% individuals) (Trepo *et al.* 2014).

The liver injury and replication of the virus can be mediated immunologically and viral particles do not cause cytopathic effect directly. Innate immunity is associated with pathogenesis of HBV infection (Trepo *et al.* 2014; Ganem and Prince 2004). Adoptive immune responses is critical for the resolution of acute HBV infection by accelerated T-cell mediated immune responses to the epitopes of HBV, however overall T-cell mediated immune response is weaker in chronic HBV infection.

The variation of genes engaged in immune system could provide protection or susceptibility against HBV infection. Uptill now several studies confirmed the relationship between persistence of HBV and genetic polymorphisms. These studies mainly focus on spontaneously recovered patients and chronic carriers, targeting 7 genes of immune system, including tumor necrosis factor alpha (TNF-alpha) (Xia *et al.* 2011; Shi *et al.* 2012), Signal activators and transducers of transcription 4 (STATA4) (Liao *et al.*2014), proteins associated with cytotoxic T-lymphocyte 4 (CTLA-4) (Xu *et al.* 2013), interferon-gamma (IFN-gamma) (Tang *et al.* 2014), Interferon lambda 3/4 (IFN 3/4) (Lee *et al.* 2014), and Interleukin-10 (IL-10) (Ren *et al.* 2015; Shu *et al.* 2015). Studies have shown significant role of these genes, whereas role of their polymorphism in chronic infection of HBV is not fully understood.

It was postulated that the effects of antiviral therapy is significantly influenced by viral and host factors (Buster *et al.*2009). Genome-wide studies revealed that the polymorphisms (rs12980275, rs12979860 and rs8099917) of Interferon lambda 3 (*IFNL3)* gene are directly linked to the effectiveness of anti-HCV therapy and the natural HCV clearance (Thomas *et al.* 2009; Suppiah *et al.* 2009). Even though its exact molecular mechanism is still unknown, same is the case with CHB. While other studies revealed that polymorphisms are the major predictors of serological response to Interferon-gamma (Sonnevel *et al.* 2011; Lampertico *et al.* 2013) whereas conflicting results have also been reported (Tseng *et al.* 2011).

Around 60% cases of HCC are linked with chronic hepatitis B (CHB) infection and in many cases HCC can result in cirrhosis of liver (Perz*et al.* 2006; Lai *et al.* 2003). HBV infection and its development towards liver cirrhosis (LC) and HCC are predisposed by certain factors such as host, environment and viral load (Thursz 2001). The data of genome-wide association studies (GWAS) have recently identified signal transducer and activator of transcription 4 (*STAT4*) a new genetic variant which is directly or indirectly linked with HBV related liver disease (Jiang *et al.* 2013). However, the predominant role of these variants is not properly explored yet. Cytogenetic location of *STAT4* is on longer q arm, chromosome 2q32.3 and it encrypts key transcription factor (Zhong *et al.* 1994). Various different cytokines like (IFN)-α/β and interleukin (IL)-2 are considered to be good activator of *STAT4* (Nguyen *et al.* 2002), for signal transmission of downstream target genes. Binding of IL-12 to its receptors on T cells and natural killer (NK) cells results in phosphorylation of *STAT4,* which in turn results in production of lamda IFN-γ by these immune cells (Wang *et al.* 2015).

A unique and complex interplay occurs between immune response, whereas the outcome of infection is shaped by viral and host genetic factors (Vanwolleghem *et al.* 2015). Our understanding towards the entire course of viral hepatitis infection that is influenced by genetic factors is increased by genome wide association studies (GWAS) (Eslam and George 2015; Romero-Gomez *et al.* 2011).It has been reviewed recently that host genetic milieu significantly contributes to the risk of HBV persistence (Thursz *et al.* 2011). It is also reported that Single nucleotide polymorphism (SNP) rs7574865 which is located on

the 3rd intron of STAT4 gene is associated to HCC risk. Studies demonstrated that the whole spectrum of HBV infection is highly influenced by this polymorphism that includes risk of persistence of HBV, reaction towards IFN-α based therapy and liver disease progression to cirrhosis (Lu *et al.* 2015; Jiang *et al.* 2016). Though, the role of this variant has not been investigated in Pakistani HBV-infected populations. Besides, multiple landmark GWAS in the *IFNL3/IFNL4* region have been recently implicated in hepatitis C and later was revealed to be linked with risk of liver fibrosis progression and HCC risk (Tanaka *et al.* 2009; Ge *et al.* 2009). While, similar role in HBV infection is still less clear and is virtually unknown in Pakistani HBV-infected populations.

The polymorphism, allelic frequencies, haplotype distribution and effect size varies significantly according to the studies on different populations. In this context, recent study suggest that in Caucasian patients infected with chronic hepatitis C *STAT4* rs7574865 is not linked to treatment specifically interferon-based or spontaneous clearance (Asimakopoulos *et al.* 2016). Therefore effect of polymorphism may vary in different population background. We investigated role of *STAT4* rs7574865 and *IFNL3* rs12979860 in HBV persistence and risk in Pakistani population.

Moreover, in developed countries more than 30% of the adults are individuals with non-alcoholic fatty liver disease (NAFLD). Additionally, environmental risk factors, certain common factors such as increase in caloric diet and inactive lifestyle are predicted to contribute in fatty liver disease in coming years (Marchesini *et al.* 2016).

Genetic predisposition is also known to be involved in moderating the degree of steatosis and liver injury (Anstee and Day 2015). Theoretically, hepatic steatosis is known to be linked to the traits that are ruled by multiple modifications and uncertain effects. According to the research studies two main missense SNPs are involved in this genetic predisposition at transmembrane 6 superfamily member 2 (*TM6SF2*p.E167K and patatin-like phospholipase domain-containing protein 3 (*PNPLA3)* p.I148M. These polymorphisms were found in either exome-wide (Romeo *et al.* 2008) or genome wide (Kozlitina *et al.* 2014) association studies on fatty liver patients. The gene patatin-like phospholipase (PNPLA3) domain (having 3, a.k.a. adiponutrin) p.I148M polymorphism

is a risk factor for fibrosis and increased accumulation of fat (Krawczyk *et al.* 2013; Sookoin and Pirola 2011). The steatosis was reported in several other studies, associated with liver scarring. Likewise, PNPLA3 was demonstrated through meta-analysis in individuals with chronic HCV infection and alcoholics (Salameh *et al.* 2015). However, the association of *TM6SF2* with the liver injuries is not that much convincing. Furthermore, few reports are published on the investigation of TM6SF2 risk genotype in HCV and NAFLD. Study demonstrated that carriers of respective minor allele are more likely at risk of developing fibrosis and steatosis (Liu *et al.* 2014).

The *TM6SF2 and PNPLA3* variants are related to metabolically silent NAFLD, patients with risk genotype are vulnerable to the development of NAFLD and the extreme forms even without depicting the characteristics that are generally accompanied with fatty liver (Zhou *et al.* 2015). Various genetic studies failed to identify ambivalent evidence to determine the association between the variants *PNPLA3* and *TM6SF2* for traits like insulin resistance, obesity or hyper lipedema. Furthermore recently at University of Sydney (CAUL) the Membrane bound O-acyltransferase domain-containing protein 7 (*MBOAT7)* polymorphism rs641738 was identified as the new risk element for NAFLD (Mancina *et al.* 2016). It was also linked with higher degree of fibrosis in alcoholic liver disease (Buch *et al.* 2015) and in HCV infection (Thabet *et al.* 2016).

To differentiate between non-alcoholic fatty liver (NAFL) and non-alcoholic steato-hepatitis (NASH) various advance procedures and methods have been developed. To clarify the role of NAFLD genetic susceptibility, a genetic analysis was performed in fatty liver patient's cohort and signs of liver injury with consolidation and deportment of *TM6SF2 (*p.E67K), *MBOAT7 (*rs641738), and *PNPLA3 (*p.I148M) variants. The frequencies within these variants were found to be associated with circulating markers. Patient analysis of genotype-phenotype interaction were performed within certain groups to evaluate PNPLA3, *MBOAT7* and *TM6SF2* variant effects on fibrosis and hepatic steatosis with an emphasis on a concept that they play a key role in progression of NAFLD.

The accumulation of ectopic lipid is related to an increase in prevalence of obesity (Ng *et al.* 2013; Lumeng and Saltiel 2011) that results in organ dysfunction, mortality and morbidity (Adams *et al.* 2005). The deposition of hepatic fat is significantly associated with metabolic complications of obesity rather than deposition of visceral fat. So, steatosis is mostly observed as a histological abnormality beyond the range of all liver diseases (Gordon *et al.* 2005) therefore, heritability of steatosis is of great concern, with genome-wide associated studies (Romeo *et al.* 2008). Nonsynonymous single nucleotide polymorphism (SNP) has been described in gene of phospholipase domain, 3 (PNPLA3) rs738409 that correlated with steatosis as well as with fibrosis in the NAFLD (Speliots *et al.* 2011).

Additionally, genome-wide associated study on meta-analysis in NAFLD on European ancestry on 7176 individuals was conducted using single nucleotide polymorphisms (2.4 million) imputed to HapMap with some additional variants at five different loci having multiples genes of (NCAN/TM6SF2/CILP2/PBX4) which can influenced by steatosis (Valenti *et al.*2010). Another study with genome-wide association of exome and functional analysis of rs58542926 C>T revealed variant in TM6SF2 (encode E167K a substitute amino acid) a causative variant of GWAS signal, and a major cause of fatty liver in NAFLD (Liu *et al.* 2014; Sookian *et al.* 2015) Interestingly, p.Gly17Glu, rs641738 C/T (missense variant) present in exon 1 of TMC4 gene (transmembrane channel-like 4) and the intergenic downstream of gene MBOAT7 are known as a risk of NAFLD (OR ~1.37), fibrosis and NASH development. These results are based on the study conducted on European descendent patients (Mancina *et al.* 2016). However, the rs641738 and NAFLD correlation has not been replicated in any other population with large groups (Luukkonen *et al.*2016). A marginal effect of rs641738 on liver fibroses without any significant effect on liver function has been reported in a case study conducted in Germany which includes relatively larger sample size (n = 515) of NAFLD patients.

Hepatitis B has an intimate link with hepatic lipid metabolism. In humans an inverse relationship has been identified in prevalence of fatty liver and positive status of HBs-Ag. It has also been demonstrated that there occur moderate to severe hepatic steatosis and

also it is associated with additional threefold HBs-Ag sero-clearance than those without hepatic steatosis (Chu *et al.* 2007). Similar observation was reported in mouse models and *in-vitro* studies (Hu *et al.* 2018; Yasumoto *et al.* 2017). Patatin-like domains (nonsynonymous coding variants) containing PNPLA3 and TM6SF2 has been demonstrated to significantly associate with steatosis in NAFLD patients (Eslam *et al.* 2016; Kozlitina *et al.* 2014).

However, limited information is available showing impact of these variants on patients with hepatic steatosis particularly in Asian population having HBV infection (Vigano *et al.* 2013). Notably, it was suggested that these polymorphisms are associated with HBV-DNA levels, suggesting a potential role in HBV persistence (Pan *et al.* 2015). Genome wide association study showed that genes (SNP) (the rs641738) of *(MBOAT7)* with SNP in the membrane bound domain of O-acyltransferase is highly found to be linked with alcohol related cirrhosis (Buch *et al.* 2015). This is the first and among very few genetic studies conducted in Pakistan addressing the role of host genetic factors *STAT4, IFNL3 PNPLA3, TM6SF2* and *MBOAT7*in hepatic steatosis and hepatitis B persistence in Pakistani population.

1.1 Aims and Objectives

Hypothesis

The host genetic variants are associated with risk of steatosis and chronic HBV persistence. Single nucleotide polymorphism in the host genes like STAT4, IFNL3, TM6SF2, PNPLA3, and MBOAT7 can influence the outcomes of HBV infection and steatosis.

Aims and Objectives

The aims of this study were to analyze seroprevalence of HBV and to investigate role of *STAT4, IFNL3,* and *TM6SF2, PNPLA3,* MBOAT7 genetic variation associated with chronic HBV persistence and hepatic steatosis in patients and spontaneously recovered individuals belonging to Pakistani population.

Objectives

Major study objectives included;

- To determine demographic information, disease history, patient signs and symptoms and risk factors for HBV related disease development in recruited patients.
- To measure the prevalence of HBV co-infection with HDV and HCV.
- To find gender based allelic and genotypic variations and their role in HBV infection
- To analyze the association of *STAT4, IFNL3* genetic polymorphism in risk of chronic HBV persistence in Pakistani population.
- To elucidate the association of *PNPLA3, TM6SF and MBOAT7* gene polymorphism with persistence of HBV infection and hepatic steatosis.
- To correlate *STAT4, IFNL3, PNPLA3, TM6SF and MBOAT7* genotypes with different clinical variables.

2. Literature Review

2.1 History of HBV

There are different theories about HBV origin but Bollkyky in 1998 suggested that this virus was originated in America. It was speculated that the virus spread via contact of Native Americans with Europeans during the last 400 years (Bollyky et al. 1998). However, this notion cannot be proven on scientific basis and today, HBV genotypes are found across various geographical regions around the world and even among non-human primate species. Secondly, the presences of HBV (ancient) in Korean mummy from 16th century also oppose this theory (Kahila Bar-Gal *et al.* 2012). Another theory about HBV origin is called Cospeciation theory, according to this, during the last 10 to 35 million years Hepadnaviridae have evolved along with their host or primate specific species. This model is affirmed by the time differences inferred between the diverse mammals and avian Hepadnaviridae. The similarity of Hepadnaviridae to those inferred in between their particular hosts is relying on fossil-based evidence as well as phylogeny (MacDonald *et al.* 2000).

The co-specification of HBV is further supported by the fact that chimpanzee HBV constitutes a unique phylogenetic clad from multiple geographic regions (Hu *et al.* 2001). However, the genome sequence of HBV from gorillas showed similarity to those of chimpanzees and also demonstrated some overlaps in phylogeny. This study posed some new questiond and contradicts already available theories. Third theory known as the co-evolution theory was proposed by Maginus and Norder on origin of HBV. This theory demonstrated that the co-evolution of human HBV is the result of migration from Africa about a thousand years ago (Magnius and Norder 1995). Although the current HBV geographical spread of genotypes can be justified by this explanation, it doesn't match well with genetic relationships between human HBV and HBV of primate origin. The predominant exposure of Americans to genotype F and presence of genotype B and C in Northeast-Asians areas is another objection on this theory. Hence, no evidences were found in support of "Out of Africa" concept in current studies. Paraskevis and colleagues reportrd phylogenetic investigation of HBs-Ag gene sequence, that evolved 22000 and 47000 years before HBV jumped into humans from an unknown source. This may

support the concept that the infection in nonhuman primates was received from humans in Africa, Asia and rest of the world (Paraskevis *et al.* 2013).

Another theory known as cross-species transmission demonstrated that close relationship of HBV in human and non-human primate was due to cross species transmission. The theory was supported by the fact that higher HBV transmissions among humans and nonhuman primates occurred in highly endemic regions. There are number of studies available to support HBV cross species transmission. Some of the evidence include HBV isolation from chimpanzee, identification of HBV recombinants in human and primates, confirmation of Mauritian macaques HBV infection, and isolation of gibbon variant from chimpanzee, all these lend a substantial credence to this model (Hu *et al.*2001; Takahashi *et al.* 2000), (Simmonds and Midgley 2005), (Dupinay *et al.* 2013), (Grethe *et al.* 2000).

Recently, Bat Origin Theory (BOT) proposed by Drexler *et al.* 2014) answered many questions after detecting Hepadnaviridae in different bat species. Bat origin of hepadnaviruses from some primates suggested unexpected absences of these viruses in other primate species, like cercopithecoid monkeys and non-Simiformes monkeys (Drexler *et al.* 2013). Despite many studies overall it is difficult to conclude evolutionary history of HBV, however anwser lies in co-species evolution based on multiple hosts including rodents, bats and even birds followed by spillover events similar to those observed in COVID-19 pandemic.

1.2 HBV virology

HBV virion is spherical in shape with diameter ranging between 40-45 nm, visualized with the help of transmission electron microscope for the first time and was named as Dane particles (Dane, *et al.*1970). The detailed structure and the shape of this virus was studied in last two decades using cryo-EM (Dryden *et al.* 2006; Seitz *et al.* 2007). It has an outer envelope that consists of surface proteins and surrounds a capsid which is icosahedral in shape. It encloses the double stranded DNA. The whole virion is very infectious anda single particle has the ability to initiate infection in chimpanzee (Asabe *et al.* 2009). During infection, other interesting fact is the production of 100 to 100,000 fold of noninfectious viral particles without viral genome. These sub-viral particles which are

non-infectious do have the filaments and can be seen as classical sphere shaped entities having a diameter of 22 nm. These particles contain only outer layer of envelope and empty virions (Ning *et al.*2011; Luckenbaugh *et al.*2015) .

2.2.1 HBV Genotypes

There are almost eight known HBV genotypes, comprising from A-H (A, B, C, D, E, F, G, H). The two dominant genotypes of HBV in Europe are A and D, while genotype F and E are more common in Africa. Asian populations are susceptible to genotype B and C. In addition, 24 HBV subtypes have been reported in different regions.

As an illustration, there are two subtypes of genotype A, Aa (A1) in Asia and Africa and Ae (A2) in North America and Europe. Depending on regional differences and distributions, there are several subtypes and genotypes. These different genotypes and subtypes show no significant clinical distinction. The rapid development and progression of HCC is highly associated with genotype C (Chan *et al.*2004). Genotype A and B affected patients show better treatment response by interferon alpha when compared to the individuals effected by C and D genotypes (Janssen *et al.*2005). Because of limited genotyping, HBV is not followed firmly by currently recommended guidelines or criteria. Even though different genotypes have been linked by response to INF, however –ve or +ve value have not been very high and other predictors i.e (HBs-Ag kinetics, HBV DNA <10 8 IU/ml, ALT >2–5× ULN,) also existed (Arends, *et al.*2013) .

2.3 Chemistry of HBV DNA and proteins

2.3.1 Viral genomic DNA

All hepadnaviruses consists of specific DNA structure (Mason 2015; Tu *et al.*2017). DNA is of smaller size 3.0 kbp to 3.2 kbp, enclosed in circular manner. A complementarity of 60-200 nucleotide exists on 5′ side of DHBV and HBV. There is no covalent bond association in this relaxed circular DNA (rcDNA) strands, though the negative complimentary strand (pgRNA) has short terminal redundancy (ca. 9 nt). The

positive strand which terminates hundreds of nucleotide away at 3′ ends is heterogeneous in length. The terminal protein (TP) linked to the rcDNA via covalent bond acts as reverse transcriptase and helps prime negative DNA synthesis (Datta *et al.*2012, Caligiuri *et al.*2016; Yin *et al.* 2017). This TP is furthered capped with long RNA oligomers (18 nucleotides), attached to 5′ end of positive DNA, results in initiation of positive strand synthesis (Watanabe *et al.*2017).

Four different classes of viral messenger RNA molecules are capped at 5′ end and are polyadenylated at 3′ side. Moreover, genomic PreC/C mRNA with 3.5 kb length is longer than the DNA template (Guo *et al.*2016). The approximate length of PreS1, PreS2/S, and X mRNAs sub-genomics are 2.4 kb, 2.1 kb, and 0.7 kb respectively. At 3′ end all the viral mRNAs carry identical sequence illustrated by shortest XmRNA. The synthesis is terminated with single polyadenylation signals by all these mRNAs. The four different promotors' initiate the transcription of four different mRNA that codes for core proteins, PreS1, PreS2/S and X promoters respectively, while regulation of all these expressions occurs by two enhancers. Enhancer 1 is located at upstream which overlaps X promoter while enhancer II is positioned slightly further to the core promoter region (McNaughton *et al.*2019).

From these viral mRNAs, seven different proteins are formed using four open reading frames (ORFs). Using the same ORF, the capsid or core protein is composed of PreC/C mRNAs, while P protein is synthesized by using an alternative ORF. The short fragments of these genomic RNAs known as pregenomic RNA (pgRNA) also act as a template during replication to synthesize rcDNA by reverse transcription (Fujimoto *et al.* 2016). The large, medium and small envelope and X proteins are coded by PreS1, PreS2/S, and X mRNAs and are regulated by single ORF embedded within the open reading frame. (Jianming *et al.*2015). Notably, 3′ end of C and 5′ end of X gene are overlapped by P gene. In addition to this pattern, all other elements of transcription including promoters, enhancers and poly A tail are also overlapped with protein coding sequences. Overall, the hepadnaviruses genomic structures were studied and characterized very precisely (Hama *et al.*2018; Lei Li *et al.*2017).

2.3.2 Viral proteins

2.3.2.1 Envelope Proteins

HBV envelope protein consists of three segments called small S, middle M and large L regions. The protein S is of 226aa residues long and other three have identical C-terminal ends, whereas the M protein contains N-terminal extension Pres2 region comprises of 55 residues. The third protein is L which is the longest one, containing PreS1 region around 108-119 residues long. During the infection, the envelope protein is also secreted to the blood stream in different forms like filaments and spheres in the absence of genome or capsid. Envelop antigen detection in blood can be used for the diagnosis of different stages of HBV infection. The significance of these proteins was found during the discovery of HBs-Ag which is crucial in diagnosis.

The S and M proteins appear in spheres, while the L-proteins usually filaments enrich virion particles (Jianming Hu and Liu 2017, Ning *et al.* 2018). The PreS1 region of the L protein consists of some determinants and are required for both envelope and capsid development during virion formation. These proteins also facilitate viral entry via receptor binding domian (Bruss 2007). The multiple function of PreS1 is due to its dynamic topology (Selzer and Zlotnick 2015). After translation process in endoplasmic reticulum the PreS1 is found on the cytosolic side and enables capsid into virus formation. Approximately, 50% of the PreS1 forms in interior and exterior regions of virus and is then translocated that allows its binding to cell surface receptors (Abraham and Loeb 2007).

2.3.2.2 The C Protein and e Antigen

As we know that the C-terminal domain or protein (CTD) is 183-185 residues which is further divided into structural as well as functional domains. While the N-terminal domain (NTD) is 140 residue long it plays significant role in capsid initiation and viral assembly (Roderfeld and Roeb 2015). The CTD is essential for proper assembly of capsid however it plays a significant role in packaging of pgRNA to nucleocapsids (NCs). The CTD also plays role in reverse transcription of viral pgRNA to rcDNA. The

two capsids which are morphological isomers with either 90 dimers (*T*3) or 120 (the major isomer *T*4) are synthesized (Pairan and Bruss 2009). The functional role of the dichotomy is still unclear. The CTD is arginine rich, basic in nature with nonspecific binding capacity to nucleic acid (Lambert and Prange 2001). Several nuclear localization signals (NLSs) (Li *et al.*2010) are also present which are important for NCs delivery to the nucleus (Gallucci and Kann 2017).

CTD in mammalian cells is heavily phosphorylated exhibiting ser-pro motifs for all three major sites of phosphorylation (Hui *et al.*1999; Li *et al.* 2015) which plays an important role in viral replication using the host protein kinases (SRPK) and cyclin dependent protein kinases 2 (CDK2) (Daub *et al.* 2002; Lai and Su 2017; Diab *et al.* 2017). These CTD and CDK2 interaction at Ser-Pro sites incorporated in capsid proteins helps in phosphorylation (Ludgate *et al.*2012). Phosphorylation of CTD is highly complex, dynamic, rapid and dramatic. CTD de-phosphorylation is reported to complement viral DNA foundation in NCs of DHBV. The phosphatases in cells responsible for de-phosphorylation of C are still unclear (Jie *et al.* 2018).

The PreC proteins (translated from PreC mRNA) are different. The sequence of both C and PreC is same however, PreC have an additional 29 amino acids residues at the N-terminal region (Zhang *et al.* 2010). The PreC is entirely dispensable for viral replication just like C protein and therefore the mutants were not able to express these proteins during infection (Martinot-Peignoux *et al.* 2016). A secretion signal is present in PreCa first 19 amino acid residues which are responsible for its translocation into lumen of endoplasmic reticulum. These signals are disrupted upon interaction with cell peptidase enzymes. The PreC residues continue proteolytic processing to eliminate the highly basic CTD in host secretory pathway, ultimately heterogeneous secretion of dimeric and soluble protein occurs. The PreC/HBeAg seems dispensable for viral replication but its presence is highly associated to play key role in the development and persistence of infection *in-vivo* and regulation of host immune system in response to immunogenic C protein (Heermann *et al.* 1984). In addition, HBeAg presence in serum is linked to increasing level of viral replication and has been evaluated as a potential marker for

screening and replication of viruses. The loss of HBeAg is generally associated with the reduction of viral load or replication (Beaumont *et al.* 2013) .

2.3.2.3 The Reverse Transcriptase

The RT or HBV P protein is 832-845 residues long, multifunctional dependence on type of viral strain plays a necessary role in viral replication. This protein is divided in to four domains separately form N-terminus (Toh *et al.* 1983). The invariant Tyr residues anchored in TP are important for initiating reverse transcription (Aragri *et al.* 2016) , and for pgRNA specific binding in presence of RT domain, which facilitates encapsidation in to NCs. The packaging of pgRNA also involves RNase H domain (Zhang *et al.*2019). The spacer region is known to be the least conserved part with four domains and thought to be associated for all mysterious functions of P. The polymerase active sites which are important for DNA polymerizations are anchored in RT domains, predominantly the Tyr-Met-Asp-Asp motif unchanged among majority of RT proteins together with those in retrotransposons and retroviruses(Li *et al.*2019).

The pgRNA template is degraded by RNase H domain during (-) strand DNA synthesis (Ko *et al.*2012). The activity of HBV DNA polymerase was discovered using endogenous polymerase assay (Xu and Ou 2004), it is also noted that the activity of DNA polymerase in the virions was evaluated for DNA synthesis with the help of DNA of virion as template. In later decades, Mason and Summers shaped new discovery that replication of DNA viruses occurs through reverse transcription using an intermediate RNA (Marugán and Garzón 2009). However, it has been proven that the biochemical study of this important enzyme is still very difficult, and no high-resolution structures of P are yet available. The discovery that a specific host factor is required for the folding and function of P offers a partial explanation to this issue (Tu *et al.*2018).

2.3.2.4 The X Protein

The Hepatitis B X protein (HBx) is 154 residues long and smallest of all the reported HBV protein and its function is least understood. There is a general conception of the X protein and believed to be involved in viral replication and also contribute perhaps to the

viral pathogenesis (Maguire *et al.*,1991). The function of X has been reported for the viral and host gene regulation and expression, Ca^{2+} signaling, DNA damage repair, apoptosis and autophagy in several reports (Niu *et al.*2017). There is no report to demonstrate the DNA binding activity of X and is presumed to involve in gene expression via protein interactions within the host cell, which are probably important for numerous viral or cellular affects driven by X. It remains to be investigated to find association related to various activities of X and their role in relation to viral pathogenesis and/or replication (esp., hepatic cellular carcinoma development) (Livingston *et al.*2017).

Due to the experimental limitation function of X protein is not well understood. Other possible reason could be regulatory or indirect role of X in viral replication (C. Liu *et al.*2017). Recent development in diagnostics and in-depth understanding of viral replication mechanisms and other relevant physiological systems will optimistically help to clarify the HBx function in viral pathogenesis and replication (Geng *et al.*2015).

2.4 Natural history of HBV infection

HBV originators can be traced back since 82 million years till Mesozoic, found as one of them integrated in prehistoric birds genome likely to be considered as one of the host ancestral of *Hepadnaviridae* (Suh *et al.*2013). Analyzing hepadnavirus DNA of endogenous avian in the genome of zebra finches the evolutionary origin has been studied (Gilbert and Feschotte 2010). It has been suggested through phylogenetic analysis and several other studies the existence of HBV in human is 336000 years old and its co-migration is being anticipated with human populations subsequently. Using the laparoscopic biopsy of liver in Korean mummy, the oldest virus isolated so far from sixteen century before (Woolhouse *et al.*2012). In early childhood perinatal infection or horizontal infection is the leading route of HBV transmission in high-endemic areas. Due to high-risk sexual activity and injecting drug use, hepatitis B is mainly a disease of adolescents and adults. There are four stages of HBV infection which reflect different level of infection severity and dynamic changes in infected host. The characteristic of each phase has already been explained in previous section. More than 780,000 deaths occur each year as reported by WHO in 2019.

2.5 Acute HBV infection

Acute hepatitis infection is symptomatic with clinical features like jaundice, fatigue, abdominal pain, nausea and vomiting (Butler *et al.*2018). In most of the cases however the patient's initially is asymptomatic in correspond to the fact of high viremia titer in body i.e. about 10^{10} virions per milliliter (Roberts *et al.*2016) with an infection rate of 75 to 100% of all hepatic cells (Harris 2016). At this early stage of infection interestingly, the innate immune system does not show any response to virus. During this acute stage of infection indeed lack of interferon response has been demonstrated in humans and chimpanzees (Barbara *et al.* 2015). These features led to in-depth HBV description as a stealth virus. Multiple strategies have been designated for early immune evasion of HBV i.e. the invisibility of viral replication process that make the host cell silence for any response against the virus thereby beating the innate sensing machinery. Moreover, interleukin 10 an immunosuppressive cytokine is also nurtured (Isogawa and Tanaka 2015).

2.6 Chronic HBV infection

The chronic HBV infection is a state with vibrant changes of disease condition. The viral titer or load and other clinical manifestation and serological parameters may switch over time (Chen *et al.* 2016). Most of the carriers of HBV at birth or after a while have normal ALT and e Antigen positive. They retain this carrier's state till adulthood.The chronic HBV inactive phase is normally characterized by the presence of anti-HBe and HBsAg, normal aminotransferase level, mild to inactive form of liver disease, low or almost undetectable HBV DNA in serum and this stage can prolong and may sustain for decades (Liu *et al.*2016). The HBsAg average clearance rate per person is 0.5% per year (Michael Nassal 2015). However, the reactivation of HBV and more rapid or aggressive replication followed by inflammatory disorders in hepatocytes can happen any time subsequently results in immunosuppression i.e. HIV infection, autoimmune disorders, cancer, transplantation and corticosteroids treatment (Buti *et al.*2015).

Cirrhosis, liver fibroses and HCC are the long term complications in chronic HBV infection. Cirrhosis is almost developed in 20% of chronic infected individuals and their

risk of HCC is 100 times more as compared to healthy controls (Terrault *et al.*2016). The HCC development risk is further elevated by decompensated cirrhosis, alcohol, age, sex, aflatoxin exposure, genotype of HBV or any other co-infection with HCV or HIV (Bertoletti and Kennedy 2015).

In individuals with occult HBV infection (OBI) which is characterized by HBV DNA presence in patient liver that already demonstrated HBsAg negative results, reactivation can be observed (Zobeiri 2013). The stable cccDNA molecules in such patients remain in hepatic cells but halted the viral replication by either genetics, epigenetics and by means of co- or post transcriptional mechanisms. The multi specific T-cell responses is also one of the interesting phenomenon that can halt the viral replication (Bhatti *et al.*2007). Significantly, the fully replication competent cccDNA molecules can lead to acute HBV infection in recipients with liver transplant from OBI-positive donors (Said 2011).

2.7 HBV correlation with HCC and fibrosis

The high risk of HCC progression and chronic persistence in HBV infection is markedly linked with multiple factors like sex, age, alcohol consumption, time of infection NAFLD, HBV DNA level, any co-infection like HIV, HDV, HCV and genotypes of virus like HBV genotype Aa and C (Xie 2017). The long term and complex interaction in-between the host and viruses is observed to demonstrate induction in HBV infection persistence. The host metabolism can be hijacked by the virus to acquire energy for proliferation and other cellular activities (Arzumanyan *et al.*2013). The dysfunction in mitochondrial respiratory chain and overproduction of oxides responsible for oxidative stress can occurs as result of this process thereby reflecting pro-oxidant/antioxidant redox imbalance. The persistence of oxidative stress and other cellular mechanisms that can lead to damage in protein or cellular membranes are considered to be potential markers in chronic HBV infection. All these are thought to be highly linked with different liver diseases like liver fibrosis, HCC, cirrhosis.

The different intracellular enzymes like xanthan oxidase, peroxisome, cytochrome p450 and other inflammatory cell activation processes are the major sources for

mitochondrial damage posed by oxidative stress. Different antioxidant defensive mechanisms are present, regulated by multiple liver enzymes like superoxide dismutase (SOD), glutathione peroxidase (GPx)/reductase (GRed), catalase (CAT), peroxiredoxin (Prx) and paraoxonase 1. They function as to quench the superoxide's and other active or instable form of oxygen to prevent the cellular activity(Friedman and Siewe 2018).

The chronic HBV infection natural history tails a dynamic mechanism resulted by conflict between immune system and virus which lead to the seroconversion of HBeAg to anti-Hbe trailed by HBs Ag to anti-HBS (McMahon 2009). The frequent occurrence of cirrhosis is the result of progression of fibrosis as outcome of long standing liver injury in HBe Ag seroconversion process. However, due to the diverse and dynamic nature of HBV infection majority of the patients during the disease course become inactive carriers and are observed with low transaminase levels and HBV DNA. This clinical remission induces the natural regression of fibrosis and has already been reported by many researchers (Sharma *et al.*2005; Fong *et al. 1*993).

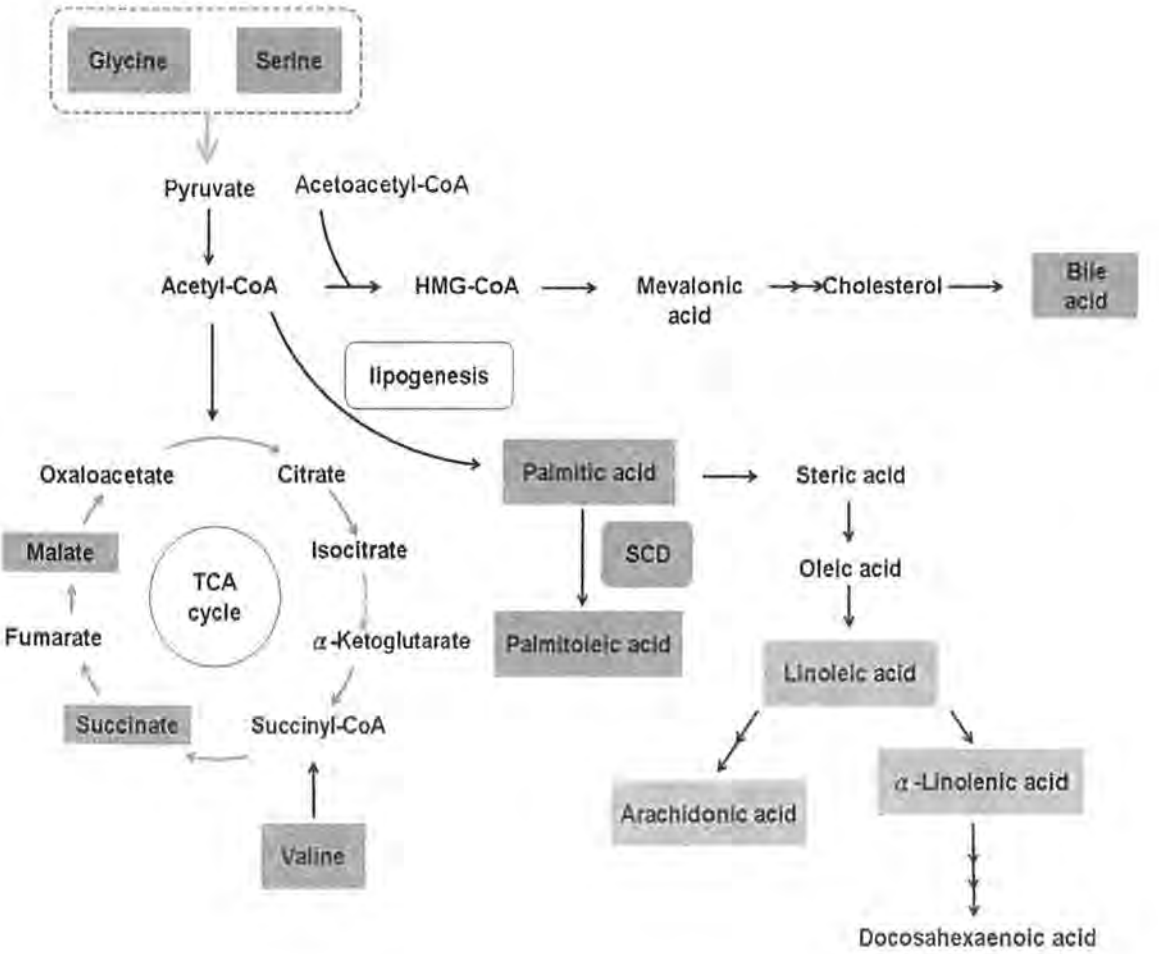


Figure 2.1: Metabolic variations in HBV associated hepatic fibrosis. The gray arrow designates the contribution of all amino acids in the specified group. Upregulated metabolites are designated in red boxes while the metabolites which downregulated are shown in blue boxes. Adapted from (M.-L. Chang and Yang 2019)

In-common, HBV with HCC occurs more frequently in non-cirrhotic liver as compare to those HCC with HCV (A. S. F. Lok 2009). This is due to the overall mutagenesis in HBV genome that integrates in host cell. The other possibility might also be due to the direct carcinogenic affect offered by HBV to hepatocytes like HBV X (HBx)(Neuveut *et al.* 2010). However, in liver if HCC has arisen where fibrosis naturally reverted from advanced liver fibrosis, might be misunderstood as having arisen from non-cirrhotic liver. It should also be kept in mind always to avoid this misinterpretation of chronic hepatitis

B infection, that might have been originated due to more advanced stage of fibrosis (Bortolotti *et al.* 2006).

Once the cirrhosis established long standing remission occurs because of the clinical profile and only thin septa is recognized. HCC developed from direct integration or from regressed fibrosis can be categorized together into "HCC from non-cirrhotic liver". All these mechanisms condense the assessment to classify fibrosis as potential risk factor for HCC. An establishment of effective surveillance system for cancer to address all the complex issues is required. In addition, to evaluate the HCC risk in non-cirrhotic and in cirrhotic liver as well the molecular markers need to be evaluated to clarify any future analysis (Ohkoshi *et al.*2016).

2.8 HBV life cycle

Protein-primed reverse transcription which is related but distinct mechanistically from replication in retroviruses is considered a hallmark of HBV replication process. Multiple genes and transformation occurs during the viral life cycle especially cis-elements and other trans-acting factors that are thought to be involved in replication (Nassal1993; Schaller 1996). Chain of events occurs during the hepadnaviruses replication.

Hepadnaviral genome replication can be broadly divided into three different phases (Figure 2.2) (1). The infectious particle contains an inner icosahedral core and a doubles stranded genome which is circular and covalently closed 3.2Kb length DNA (RC-DNA or relaxed circular); (2). The RC-DNA upon infection is converted in to a plasmid like circular and covalently closed DNA (cccDNA) inside the host cell nucleus (3). The cccDNA several different genomic and sub genomic RNAs are transcribes through RNA polymerase II. The pregenomic RNA (pgRNA) is packaged selectively in to capsid progeny and reverse transcribes by co-packaged P protein in to a new RC-DNA genome. The intracellular cccDNA amplification take place by matured RC-DNA containing nucleocapsids, which are further enveloped and release occurs as progeny virions from the cell. The genome conversion and its unique initiation mechanism with particular emphasis on reverse transcription steps are illustrated below in figure 2.2.

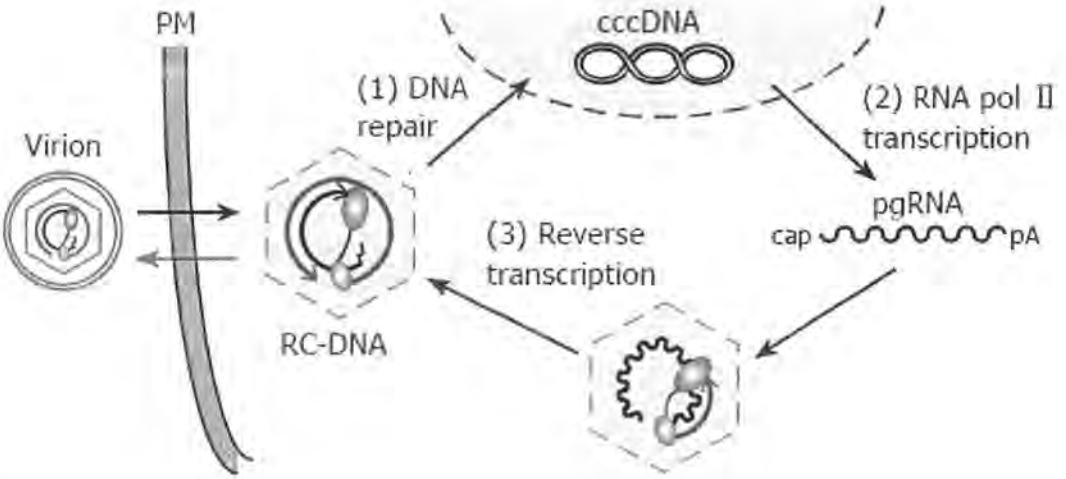


Figure 2.2: Hepadnaviral genome replication cycle where the host cell is infected by enveloped virions. After infection the RC-DNA with its nucleocapsid is release in to the cytoplasm. Transportation of RC-DNA occurs and repaired from cccDNA. Multiple transcripts pgRNA are produced by using cccDNA RNA polymerase II to initiate transcription. pgRNA together with P protein is encapsidated and in nucleocapsid is reverse transcribed. New RC-DNA is generated by (+)-DNA synthesis from the (-)-DNA template as result new cycles which leads to intracellular amplification of cccDNA. Subsequently releasing the new RC-DNA comprising of nucleocapsid and enveloped virions in to plasma membrane (Adapted from (Beck and Nassal 2007).

2.8.1 HBV entry and uncoating

The initial stage of infection is attachment of virions to the hepatocytes or cell receptors on the hepatocytes membrane. After binding HBV penetration occurs in to cell cytoplasm through AGL of HBsAg. The whole process occurs or facilitated via heparin sulfate proteoglycans (HSPG) which are associated to hepatocytes thus leading to the cellular entry (Figure 2.2) (Watashi and Wakita 2015). This co-receptor and initial step facilitate the pre-S1 receptors domains to the sodium taurocholate co-transporting polypeptide (NTCP). Ultimately this is considered a receptor entry of HBV to host (Schulze *et al.*2007).

The bile acid homeostasis is maintained normally by NTCP via enterohepatic circulation. The human gene SLC10A1 found on chromosome 14 long arm encoded NTCP. The HBV envelope protein with domain pre-S1 contain two important regions of 75 amino acids at N-terminal point end which is vital for NTCP (Sun *et al.*2017). To permit virion infectivity these sequences must be myristoylated. The receptor mediated endocytosis is crucial for viral nucleocapsid delivery in to the cytoplasm after binding (Treichel *et al.*1997; Huang *et al.*2012). The genetic material of virus is loaded in to the nucleus once the nucleocapsid is transported to the nuclear membrane. Using the host cell machinery and other important enzymes release of RC-DNA occurs and further converted in to cccDNA, which results in viral minichromosome formation.

The HBV template is used for all viral mRNA transcription and also play key role in persistence of virus (Datta *et al.*2012) (Figure 2.1). the viral polymerase protein release is the initial step in this conversion from the 5′ end of –strand and is attained by employing repair enzyme of the host DNA known as tyrosyl-DNA-phosphodiesterase 2 (TDP2) (C. Seeger and Mason 2000). The consequent cccDNA generation steps are still need to be explored.

The genomic as well as subgenomic mRNA respective transcripts are transcribed using RNA polymerase II of host from HBV minichromosome (Melegari, *et al.* 2005). All the transcripts are positive oriented, heterogeneous and polyadenylated at the 3′ capped at 5′ end. The two genomic transcript having a longer length than genomic and codes the polymerase and pre-core protein. The early transcription, translational event like generation of pre-C/C mRNA functions in translation of the pre-core protein which is further processed and secreted finally as HBeAg (Lucifora *et al.* 2018) and has not any defined role in reverse transcription. On the other hand the multifunctional pregenomic RNA (pgRNA) serve as template in reverse transcription process to negative DNA stand for translation of both HBV polymerase and HBcAg. The HBV minichromosome transcriptional activity is normally maintained by HBx, mainly offered by hyperchromatinization blockage through Smc5/6 (Christoph *et al.*2016).

2.8.2 HBV DNA Translation and replication

Following the pgRNA packaging in combine with newly translated polymerase in to subviral core particles, the genomic replication of HBV is initiated, result in cytosolic formation of replication complexes. Within the nucleocapsid of HBV process of reverse transcription take place. Once the polymerase translated off the pgRNA molecules, subsequently followed by packaging and finally the domain of terminal protein binds to the epsilon (ε) loop at the 5′ end of pgRNA to prime reverse transcription (Xi *et al.*2019). This loop is also a encapsidation signal around which assembly of cytoplasmic protein dimers in to nucleocapsid occurs. The translation completion is followed by some conformational changes in polymerases that results enzymatic activation and priming actively DNA synthesis along with terminal protein binding (Yang *et al.*2014).

Translocation of complex Pol-oligonucleotide (PolG-A-A) to the complementary sequence of direct repeats (DR-1) region occurs. The DR-1 is located to the pgRNA 3′ end, then synthesis of – DNA strand remain continues until it attains the 5′ end of the pgRNA molecule. This results in short terminal redundancy generation of nearly 8 to 9 nucleotides (Hou *et al.*2019). During the course of reverse transcription pgRNA is degraded by RNaseH. However the terminal 18 nucleotide 5′-capped having DR-1 sequence is left unaltered. This fragment contains a homology of 6 nucleotide align to the direct repeat sequence thereby allowing the minus strand circularization. The 18 nucleotide containing capped RNA is then further translocated to a second DR sequence known as DN-2 at the 5′-end of newly synthesized minus strand. Once the short nucleotide sequence is translocated it now acts as a primer for formation of plus strand. This synthesis process continues until it reach a maximum length of about 50-70% of the minus strand (Bani-Sadr *et al.*2003). The detail structure and other parameters of HBV epsilon (ε) stem-loop structure is illustrated below in figure 2.3.

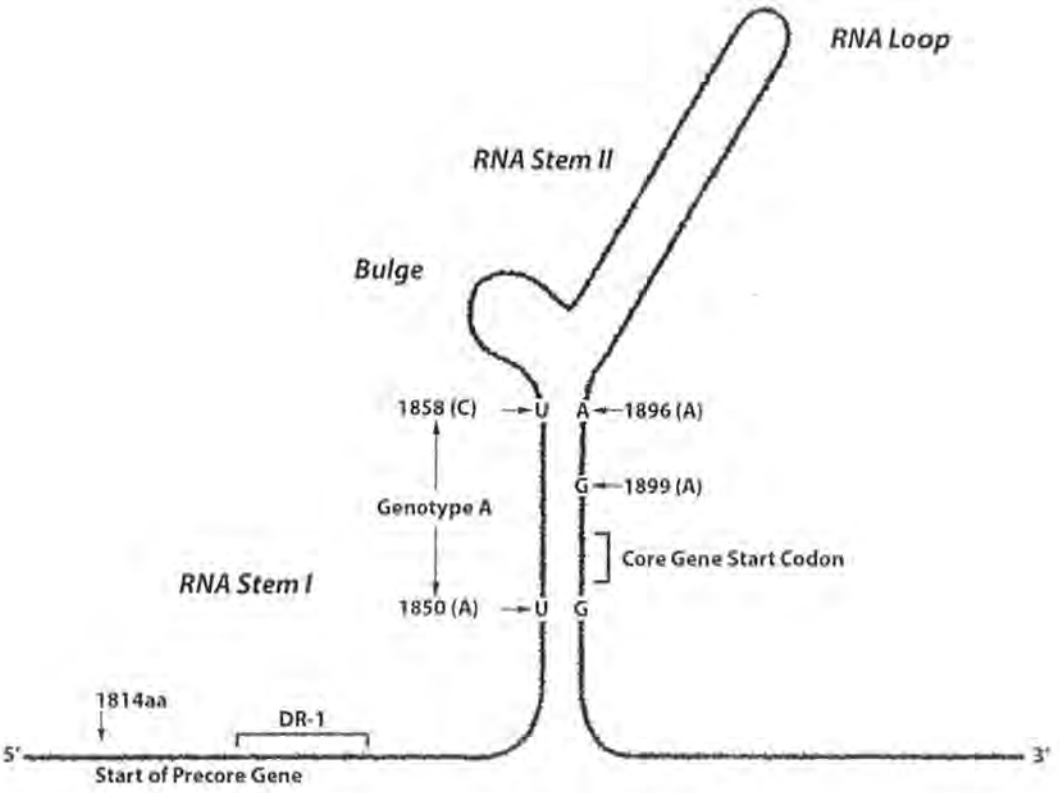


Figure 2.3: The figure shows HBV epsilon (ε) stem-loop structure which is highly unique structure within 10 HBV genotypes. The genotype A2 base change positions are also shown are the most common translational precore mutations at G1896A (precore stop codon: UAG) and G1899A. Adopted from (Wong and Locarnini 2018)

2.8.3 HBV assembly and release

The synthesis and assembly of the viral envelope and HBsAg su-bviral particles occur in endoplasmic reticulum and then bud in to the lumen of ER. The assembly and synthesis of HBc protein is attained in cytosol independently of envelope protein. The envelopment of the nucleocapsid occurs upon the phosphorylation of the nucleocapsid. This process is coupled with the minus strand synthesis initiation. The genomic RC-DNA is present in the assembled nucleocapsid and before exertion of the cell selectively become enveloped. An export bias presence towards the genome that have accomplished minus strand synthesis and also initiated the synthesis of plus strand. A critical relative molar ration of

Pre-S1 to S proteins is usually required to accomplish replication core proteins envelopment. Without this particular ration abnormal release or maturation of virions is not possible (Selzer and Zlotnick 2015). The same phenomenon occurs with many other enveloped viruses as well. The aforementioned protein is highly composed conserved structure within 10 HBV genotypes and the actual positions of base alter forgenotype A2 are shown, as the most common translational precore mutations at G1896A (precorestop codon: UAG) and G1899AD.J (Lahlali *et al.* 2018). Some studies found that the endosomal sorting complex which is vital for carrying (ESCRT). A different component of this system has a vital role in replication cycle of HBV. In particularly, the mature neucleocapsid formation which is an integral step in replication and release of virions require ESCRT-II proteins while budding of virions and egress is subjected to ESRCT-III and VPS4 ATPase presence (Kang *et al.*2019; Sun *et al.*2018).

2.9 Globalprevalence of HBV infection

HBV is one of the most sever and common viral infection occurs globally with significant morbidity and mortality (Ott *et al.*2012). HBV infect about one third of the globe population may be acute or chronic infection with clinical manifestation of fibrosis and HCCC. It has been demonstrated that about 5% of the population develop chronic infection and are most of the time are carriers (Papastergiou *et al.* 2015). Each year globally about 780,000 HBV related death occurs (Hyun Kim and Ray Kim 2018). The risk of developing HBV infection and its consequences i.e. to develop complications are inversely proportional to the infection age. The chances to develop chronic infection with subsequent complications are about 90% if the transmission occurs prenatally. In least developed and endemic countries the horizontal transmission is frequent in comparison to vertical transmission occurs in developed countries. However, in developed countries, most infections in young people occur through drug injection or high-risk sex. (Jefferies *et al* 2018).

2.10 Prevalence estimates

Data of 120 countries being used by Polaris Observatory Collaborator and it is estimated that the HBsAg global prevalence was 3.9% in 2016. The uncertainty of interval (UI) is

95% which corresponds to 291,992,000 infections. Out of these numbers only 29 million i.e. 10% were diagnosed out of which 5 % eligible for treatment and received therapy against viral infection. Moreover about 1.8 million were at age of 5 years who got infection with an estimated prevalence of 1.4%. It is reported that 87% of infants had received booster doses of vaccination of HBV in the first year of life. The timely birth dose vaccination was received by 46% and 13% had immunoglobulin against HBV along with full vaccination regime. Less than 1% of the mother who had a high viral load received anti-viral therapy to ensure the reduction in transmission of virus from mother to child (Razavi-Shearer *et al.*2018).

2.10.1 Prevalence of HBV genotypes

Prevalence of HBV genotype has reported in 2018 by Velkov and his colleague and is mainly divided in to nine different genotypes A to I, but the contribution if each genotype to the estimated 250 million chronic infections is not well understood. Velkov and his colleague carried out a literature search on the virus genotyping data globally. More than 900 publications were assessed and the extracted data from 125 countries was presented to find out the estimated chronic infection caused by each genotype per country and also to find out its distribution globally. It is estimated that 96% of the chronic HBV infection globally are induced by 5 out of 9 reported genotypes. The most common was genotype C (26%), followed by D (22%), E (18%), A (17%) and B (14%). While the genotype F to I together can cause only 2% of the total infection. This work provided an up to dated information of global chronic HBV infection in correlation to the type of genotype contribution to the global chronic HBV infection burden (Velkov *et al.*2018).

2.11 Transmission and prevention

The mucosal and percutaneous exposures are the main routes for HBV transmission to blood and other body fluids of infected individual. The transmission of the virus occurs in in multiple ways form one individual to another including perinatal route, sharing of needles, sexual and health-care worker contact. The infectious HBV with high load and concentration is generally found in serum and blood. However, other body fluids like saliva and semen can also be infectious. The major reservoirs for HBV infection

transmission are those individuals with chronic HBV although any individual showing positive results for HBsAg can transmit infection possibly by sexual contact and households.

Contaminated objects and surfaces are also the source of transmission because HBV remain viable in environment for certain period of time. Vertical transmission of chronic HBV from infected mother to infants is one of the common route for HBV globally. This transmission mostly takes place during birth, but that accounts very rare and estimated to be lesser than 2 percent of perinatal transmission. The perinatal infection risk is 5-20% in newborns babies from mother who is HBsAg positive while the risk is high around 70-90% in case of mother who is HBeAg positive (Pan *et al.*2016).

HBV transmission also occurs when there is a prolong contact with any suspect or infected individuals. Prior to the HBV immunization implementation in universal infants, in the United States it is more likely estimated that 16,000 children under the age of 10 years get infected annually through HBsAg positive household members or community contacts exposure. Certain doubts still exist about the transmission but it is hypothesized mostly the viral transmission occurs through contaminated body fluids, blood, parental exposure, or playmates that HBV inoculation occurs in to cutaneous abrasions, scratches, and other lesions on mucosal surfaces (Wilson *et al.*2018).

Sexual contact is major route for HBV transmission. Chronic HBV infected individuals have been reported with high sero-prevalence of HBV infection which is possibly due to the house-hold contacts. Moreover acute hepatitis B patients are more expected to transmit virus in multiple heterosexual partners. High rate of disease has been observed/reported in men in sexual contact with men (MSM) and the sero-prevalence rate of HBV persistence is higher in such individuals as compared to general population (Chen *et al.*2015).

Individuals with injectable or drug abuse are at higher risk and predispose to different blood-born viruses including HBV infection. These individuals doing unhygienic practices and using contaminated needle, syringes and other drug stuff or equipment's. Similarly by observing the data of 10 years, most of the individuals who are using

injections for drug abuse in the United States and elsewhere have serological indications of current or past HBV infection. Depending on the prevalence in any community the risk of chronic HBV infection among drug users may vary depending upon drug sharing and other preparation practices among individuals. Almost 70% of drug users (through injection) were HBV positive in mid 1990s at United States after 5 years of injecting. Other routes beside injection drug use and percutaneous exposure have also been studies like acupuncture and tattooing (Keane *et al.* 2016).

Globally, health care associated transmission of HBV has long been documented as key source of acute and chronic HBV. Patient to patient, Patient-to-provider or health care personnel and vice versa have been recognized as major source of transmission although the frequency at which these transmission occurs are broadly conflicting. The common transmission before the vaccination of health care workers for Hepatitis B was documented to be patient to provider. Over 12,000 infection of health care worker per year has been documented in the United States in era before HBV vaccination. Risk of the health care workers for HBV infection is highly associated with the level of risk assessment for the pathogens and also the exposure to body fluids and hospital sharps like needles. The risk for HBV infection also varies and largely dependent on the time of exposure, concentration and volume of viruses in the infectious fluids. Following the needle stick injuries the inoculation risk is at least 30% if the blood is HBeAg positive, while 6% in case of HBeAg negative blood (Pan *et al.*2017).

In developing countries, the HBV transmission from one patient to another by means of different sources is a major cause of new infection. The contaminated equipment's can result in percutaneous exposure to HBV by being using of medication and other procedures involved in patient health care, mucosal or blood exposure to contaminated medication and are considered potential threats in patient-to-patient transmission for HBV infection. Due to lack of infection prevention and control systems, awareness among health workers and other breaches in protocols for proper biosafety and biosecurity lead to equipment's contamination in health care settings and are considered major threats for developing nations in spread of HBV. Other problems associated with exposure to HBV include lacking of various resources for suitable sterilization procedure,

reusing of disposable items and equipment, and cultural partialities to prefer injections overuse. In 2000 it is estimated that over 21 million of individuals got infected with contaminated syringes worldwide that account for almost 32% of all new infections. Additionally in developed countries, outbreaks concerning this kind of transmission stay a tenacious obstacle as well, and they frequently stem from lapses by health care personnel's in infection control practice. Multidose vials, jet injection guns, finger stick devices, and acupuncture needles are other associated transmission vehicles for HBV. In healthcare setting the most frequently touch contaminated surfaces also serve reservoirs for transmission of HBV includes in particularly the dialysis units (Pan *et al.* 2016).

HBV transmission from provider to patient is rarely reported. Mostly the transmission events of HBV are largely being associated with invasive procedures undertaken by health care workers. Most of the transmissions in health care settings are observed in period before when HBV vaccination became available and also the proper implementation of precautionary measures with proper SOPs and/or protocols of guidelines for infection prevention and control (Jourdain *et al.* 2016). In most part of the world the transmission associated with body fluids like blood and sera has largely eliminated due to advancements in diagnostics, prior screening of blood and ensuring to implement other techniques to screen blood for any contaminants, also using different approaches like factor concentrates to ensure inactivation of virus in blood made products (Abdela *et al.* 2016).

Numerous effective approaches to prevent HBV transmission have been employed prior to introduction of vaccines for HBV. These measures were so effective includes blood screening of donors, plasma derived product preparation to ensure inactivation of virus implementation of infection prevention and control measures, suspected exposure followed by immunoglobulin administration for HBV, especially in case of babies born to HBsAg positive females. Despite these strategies were good enough to decrease the risk for transmission of HBV but still none of the aforementioned approach was effective as active immunization of individuals with HBV vaccine that remains most critical prevention measures for HBV transmission (Eke *et al.*2017).

2.12 HBV Vaccine

Plasma derived composed of purified HBsAg was first hepatitis B licensed vaccine. The current available vaccine for HBV is produced by recombinant DNA technology and is typically administered in a series of three doses. In the United States different age group individuals needed to receive two or four dose of vaccine. Hepatitis B vaccine composed of single antigen can be administered at the time of birth and to babies younger than forty two days. The single-antigen, concurrent with other vaccine can be administered at any age. Moreover, many other vaccine combinations for HBV virus contain HBsAg not only in the United State now but also licensed in rest of the world as prophylaxis. These vaccines are highly immunogenic to provide immunity and effectiveness can be used interchangeably when administered in a dose recommended by manufacturer with an appropriate age group (Yao *et al.*2016).

Hepatitis B licensed vaccination adherence lead to a protective anti-Hbs concentration of (≥10 mIU/ml), in 90-100% of healthy infants, children and adults. Studies also conducted to determine the efficacy of vaccine showed that almost 90-100% of fully vaccinated individuals, developed anti-HBs concentration higher or may equal to 10 mIU/ml and were protected from HBV infection preliminarily. The immunocompromised patients and adults over age of 40 are least likely to develop a good concentration that can protect. Hepatitis B vaccines are highly immunogenic and has not been indicated with any post-vaccination serologic testing in United States, except in special conditions i.e. among those born to positive HBsAg mothers, or individual with any occupational exposure to body fluids, drug abusers and immunocompromised conditions (Yao *et al.*2016). As post-exposure recommendation or using as immune-prophylaxis, the vaccine against HBV are consider highly effective and can prevent transmission through perinatal.

The effectiveness of hepatitis B vaccine followed by immunoglobulin administration with 12-24 hours right after birth has been documented to prevent both acute and chronic HBV in infants born to HBeAg and HBsAg positive women. Without administering immunoglobulin, the HBV vaccine is effective in prevention of perinatal infection, as in most cases the vaccine alone is used where the other consideration and cost make the

immunoglobulin use impractical. The key element of the post-exposure immuno-prophylaxis effectiveness for infants from mothers with HBsAg positive in initial dose on-time administration of hepatitis B immune-globulins (Ganczak *et al.*2019).

2.13 Immune Response against HBV

The replication of HBV occurs within hepatocytes without resulting any damage directly. The host develops immune response which is not only important in control of spread but also has a significant impact in establishing the inflammatory events in pathogenesis. Innate and adaptive immunity evolved to play different role in infection control.

2.13.1 Innate immune responses against HBV infection

The innate immune host response to HBV has not been well studied and also restricts the knowledge due to technical limitations. There is lack of robust HBV in-vitro infection system to establish the fact of innate immune response in HBV infection. Moreover, the in-vivo models for hepadnavirus are plagued by high costs and other ethical issues. The lack of IFN-α-mediated immune response in HBV is still controversial. The possible reason might be the escape process of HBV by cccDNA sequestration to the cell nucleus. HBV does not act in a cytopathic manner by itself and the cirrhosis, inflammation in liver and fibrosis are the host immune response consequences. As discussed the HBV during early infection evade the innate immune system and therefore adaptive immunity is considered one of the main response to the virus pathogenesis.

2.13.2 Humoral immune response

The humoral immune response is generally recognized as critical entertainer in HBV clearance. The neutralizing antibodies paly no dominant role in viral clearance at early stages and can only be detected in later stages of acute HBV infection. These antibodies are thought to form complexes with virus particle and preventing the viral spread in the body. The antibodies which provide protection from the reinfection gas been studies in the chimpanzees (Chang *et al.*1997). Furthermore, the soluble HBsAg and e antigen derived from the core protein is consider a hallmark for HBV persistence. The raised anti-HBs titer after vaccination in human is correlates with viral infection prevention. It

has been observed that the incidence of HCC and chronic infection as well reduced after the implementation of program for hepatitis B vaccination in 1984 in Taiwan (Chen *et al.*1996).

2.13.3 Cellular immune response

The role and outcomes of $CD4^+$ T cells in in HBV infection has been demonstrated in chimpanzee. Although $CD4^+$ T cells depletion in week 6 afterwards inoculation had demonstrated with no duration or any other outcome effect in acute HBV infection but however the $CD4^+$ T cells depletion prior to the virus inoculation led to disease persistence. The results revealed that primarily there is no role of $CD4^+$ T cells as effector cells but are important for other immune cell priming. The timing for priming of other effector immune cells may be important for contribution to elicit immune response and to maintain specific B and $CD8^+$ T-cell response against the virus (Gelinas *et al.*2017).

$CD8^+$ T-cell play a key role in HBV infection resolution and the these cells even emerge prior to the symptomatic liver disease development , associated with massive viral load reduction. Moreover, the basic $CD8^+$ T cells role in viral clearance has been supported by studies depletion in chimpanzee. It has been shown that $CD8^+$ T cells deprived animals could not clear the infection until these same cells reappear. The virus specific T cell response is vigorous, multi specific and poly functional in patients who clear the infection spontaneously (Hanson *et al.*1984). On the contrary, the low cytokine production like IL-2 or IFN-γ and its proliferation rate, functionally impaired and $CD8^+$ T-cell response can lead to chronic hepatitis B infection. The HBV DNA level in the body is inversely related to the impaired immune functionality. A high viral load can be observed in case of multi specific $CD8^+$ T-cell suppression.

The condition of exhaustion is characterized by week effector function, inhibitory receptors expression i.e. PD-1 and T-cell intermediate state and its differentiation (Webster *et al.*2004). Several different factors are likely involve in this impairment. Among them, IL-10 immuno-suppressant cytokine might play an important role in chronic HBV establishment. It is of worth notice that IL-10 is more likely to be highly express in chronic and acute infection. The significance is further supported by data

generated from LCMV infected clone mice in which, the cytokine blockade contributed to the virus specific $CD8^+$ T-cell restoration and further infection elimination (Yong *et al.*2015). Beside this the necessary amino acid deprivation i.e. l-arginine, induced by hepatocyte dying release arginase, impairment in signaling of T-cell receptors due to CD3ζ-chain down regulation on T cells. Further $CD8^+$ T-cell failure determinants include lack of $CD4^+$ T-cell help and inhibition of virus-specific $CD8^+$ T cells as well by other immune cells like natural killer (NK) cells (B. Rehermann *et al.*1995).

NK cells are another immune subset, considered one of the main effector cell to fight the acute and chronic HBV infection. NK not only providing adaptive immunity against HBV by modulating the dendritic and T cell but also play a vital role in innate immune response against HBV infection. NK cell are predominantly present in the liver as compared to blood. However, these are kept hypo responsive functionally and is characterized by inhibitory receptors advance expression i.e. NKG2A and loss of MHC-I binding receptors Ly49 (Zerbini *et al.*2008). The IL-10 derived from kupffer cell is shown to increase the active proposition of NK cells, studied in mice models. On the other hand, the suppression of NK cells under physiological conditions are thought to contribute liver tolerogenic milieu and to evade immune control are reported to be exploited by hepatotropic pathogens (Ferrari *et al. 1*990).

Both type of functions i.e. cytokine mediated and cytolytic are exerted by NK cells. Cytotoxicity of NK cells has been however reported sometime in chronic HBV infection. The possible reason for this might be the high rate of production of IL-10 in livers which function as immunosuppressant and also TGF TGF-β are thought to be considered with enhanced HBV inhibitory influence on the cross talk between dendritic cells of plasmacytoid and NK cells. The NK cells in the hepatocytes are mainly acting by killing the hepatic cells and thereby most of the time leads to inflammatory and other damaging effects. However, the protective effect of NK cells in liver diseases by limiting the fibrosis in hepatic cells is well documented by stellate cells killing mechanism. The dual role of NK cell in chronic HBV is underestimated furtherer more by suppression of $CD8^+$ T-cell virus specific response which can prevent viral clearance and liver inflammation both (Thimme *et al.*2003).

2.14 Association of variants with HBV and steatosis

Researcher in the field of hepatology has made great contributions by precisely pointing out how different genetic variants', phenotypes and protein scan establish prominent role in pathophysiology of liver associated diseases. They examined number of SNPs which are linked to steatosis and persistence of the infection and obtained several hypotheses. Moreover, genetic mutants have a distinctive role and its ability to distinguish patients on the basis of genotyping, to find out susceptibility of individuals to infection.

2.14.1 FABP1

This is a protein coding gene of human which main function is binding and metabolism of fatty acid. The fatty acid binding protein 1 (FABP1) is mainly expressed in liver cells and actively involved in transportation of long chain fatty acid (LCFA).Moreover this protein also performed function transportation and metabolism of phyto-cannabinoids and endo-cannabinoids and other molecules of hydrophobic nature. The metabolic conditions like obesity is highly linked to the protein altered expression (Schroeder *et al.*2016). A study findings showed the FABP1 expression in liver is not upregulated by protein HBx (Wu *et al.*2016). The upregulation mechanism occurred via HBx protein interaction with liver enriched HNF3β and C/EBPα transcription factors and another lipid sensor nuclear factor (PPARα). This whole process in turn activate the promoters of FABP1 and frequent expression of these proteins are highly correlated with and increase rate of uptake in fatty acid (Lim and Kumar 2017).

2.14.2 MBOAT7

The MBOAT7 gene is an important gen which encodes membrane bound acyltransferase family member that possess activity if acyltransferase. The lysophosphatidylinositol acyltransferase is an encoded protein with specificity for an acyl donor molecule arachidonoyl-CoA. The protein paly active role in phospholipid re-acylation and an integral part of remodeling pathway of phospholipid which is known as Land cycle. A genome wide study was performed by Buch and his colleague (Buch *et al.*2015) and evaluated influence of SNP variant rs641738 on MBOAT7 expression that can result risk

for alcohol related cirrhosis. The enzyme encoded by MBOAT7 possessacyltransferase activity for lysophosphatidylinositol and has been demonstrated with anti-inflammatory mechanisms through arachidonic acid regulation in neutrophils.

2.16.3 TM6SF2

The transmembrane 6 superfamily 2 (TM6SF2) is human gene located on chromosome 19 at 19p13.3-p12 locus precisely. It is flanked by HAPLN4 (a peptidoglycan and hyaluronan linked protein, involved in extracellular matrix formation) and SUGP1 (a G patch and SURP domain containing domain involved in splicing of pre-mRNA) genes downstream and upstream respectively. The TM6SF2 expression is only documented in the adult stage in human, particularly in the liver and intestine in moderate level and at low level embryonic tissue and ovary (Kozlitina *et al.*2014). The expression is indicated in other sources as well and includes kidney, colon, stomach, lung, brain, testis, and adipose tissue. The confocal microcopy study showed that the localization of TM6SF2 occurs in ER and the compartments of Golgi in human liver cells. The TM6SFs gene variants due to production of very low density lipo-protein (VLDL) are responsible for NAFLD. The reduced TG-rich lipoproteins secretion is found to be associated with TM6SF2 inhibition which also found to increase the lipid droplet content, and concentration of cellular TG. In contrary, the overexpression of TM6SF2 is documented to reduce steatosis in liver cells. TM6SF2 is thought to be metabolism regulator of liver fats, opposing TLRs secretion effect and hepatic droplet content (Mahdessian *et al.*2014).

2.14.4 *PNPLA3*

PNPLA3 gene plays a key role in protein making in hepatocytes and fact cells which is known as adiponutrin. The polymorphism in PNPLA3 provide genetic basis for steatosis in hepatocytes in normal population. This can also induce glucose metabolism dysregulation. The genetic susceptibility of steatosis in hepatic cells is conferred by PNPLA3 SNPs that encoding adiponutrin. Substitution of isoleucine-to-methionine by to *PNPLA3* rs738409 C>G which induce variant of I148M adiponutrin with TG reduce activity, and hydrolysis and predisposition to hepatic steatosis of normal population ((Chinese, Korean, Japanese, Indian, Filipino, Turk, Mexican, Belgian, American, and so

forth) (Baclig *et al.*2014). Except for rs738409, some other *PNPLA3* SNPs (rs139051, rs2281135, and rs2294918) are also related to high risk of steatosis in ethnic groups of East Asian, African, Mexican Americans and Caucasians (Q. Li *et al.*2012). However a study reported that predisposition of chronic hepatitis individuals to hepatic steatosis is linked to *PNPLA3* rs1010023 in population of Chinese Han (Pan *et al.*2017). Contrastively, the glucose dysregulation is prevented by *PNPLA3* rs1010023 by attenuation the resistance of insulin, probably on BMI basis reduction.

2.15 STAT4

JAK-STAT-signaling is considered one of the main mechanisms in host for mediating HBV activity. This pathway provides a critical role in viral clearance, hepatic inflammation and fibrosis as well. STAT4 which is a member of STAT protein is activated by IL-12 cytokine and IFN-α which are produced in response to viral infection, thereby regulating the tissue inflammation, anti-viral activities and fibrosis (Kong *et al.*2012). The exact STAT4 role in viral pathogenesis and injury in liver is still unknown, but knockout mice with STAT4 data revealed context dependent effects with mice being highly vulnerable to acute T cell hepatitis while an equal liver injury reported after reperfusion (Boeijen *et al.*2017). Polymorphism in STAT4 gene has been identified via genome wide association study and shown to increase the HCC risk, furthermore shown that there is an association with full spectrum of HBV outcomes in Asian patients (Li *et al.*2016).Genetic variation in STAT4 at rs7574865 is correlated with hepatic fibrosis and inflammation with high rate in Caucasian untreated patients with chronic infection of HBV. This effect is regulated by expression and phosphorylation mechanism of STAT4 that ultimately modulates NK cell driven IFNγ production differentially (Sharkawy *et al.*2018).

2.16 IFNL3

The interferon lambda 3 (*IFNL3*) gene polymorphism has been documented to associated with clearance of HCV induced with drug. The first line therapy for chronic hepatitis B is reported IFN-α but response is shown in a limited number of patients. The STAT4 genetic variants has been highly associated with HCC related to chronic HBV however

the prediction about the role of *IFNL3* polymorphism in treatment of chronic HBV infection yielded some controversial results (Origa *et al.*2015; Wei *et al.*2018). The major role of IFNL3 in CHB infection has not been fully explored but however two studies support the idea. Initially it was reported that as a reliable predictor *STAT4* rs7574865 in response to IFNα therapy in CHB viral infections for HBeAg positive patients and could be used for optimizing CHB treatment (Jiang *et al.*2016). The later study suggests that gene IFNL3 is an important predictor for CHB patient response in IFN therapy (Lin *et al.*2016).

2.17 Diagnosis of HBV infection

Anti-HBcIgM, IgG, HBeAg, anti-HBe, HBsAg, anti-HBs are considered potential serological markers for HBV infection with consideration of HBsAg as hallmark of infection. Both IgG and IgM appears within 1-2 week of the infection once HBsAg appeared in the body during an acute infection, with a raised amino-transferases and symptoms with other advanced clinical manifestation. During the chronic HBV infection, the persistence of IgG occurs while the IgM anti-HBc can be documented in some patients with a low titer as compared to that present in acute infection but with severe exacerbations (Candotti *et al.*, 2012).

The immunity to HBV infection is highly represented by the presence of anti-Hbs and is the only marker detected for HBV in the individuals' acquired immunity through vaccination. Its presence is highly associated with anti-HBc IgG in those who recovered from the HBV in past. Some individuals showed positive HBc IgG but no anti-HBs reported in patients who are HBs Ag positive, a serological pattern referred to as isolated anti-HBc. Detectable HBV DNA can be observed in the liver of most of the patients with isolated anti-HBc and had history of previous HBV exposure while some cases showed detectable serum HBV DNA. This type of presentation is termed as occult HBV infection. During chemotherapy or any other immunosuppressive therapy reactivation of the infection can be observed in HBsAg negative, anti-HBc positive individuals followed by HBsAg reappearance (Raimondo *et al.* 2008). In the past, anti-HBe has been shown to

slow down the viral replication and infectivity but its use has largely been superseded in this context by HBV DNA testing (Aftab *et al.*2018).

The replication of the virus is being shown by the HBV DNA which is directly correlated to the viral load measurement. The real time PCR is the most widely used HBV DNA assay with a detection limit of 10–20 IU/mL and a linear range of detection up to 10^9IU/mL. The concentration of HBV DNA during chronic HBV infection course can vary ranges from undetectable to high than 10^9 IU/mL.

2.17.1 HBV detection by using HBsAg

Commercial assays during the past few years have been approved to detect or quantify HBsAg concentration in many countries includes Europe and Asia. A correlation between the amount of transcription activity of cccDNA in liver and concentration of HBsAg in serum has been shown especially in positive HBeAg patients. The response to interferons is predicted by monitoring of HBsAg and also to identify individuals which has normal concentration of alanine aminotransferase and HBeAg negative with disease progression (Yeo and Chan 2013).

2.17.2 Screening test by ICT

Several multiple rapid diagnostic tools have been evaluated for the confirmation of HBsAg including immune-chromatographic kits (ICT). These are rapid and potable to detect HBV at community level with superior speed as compared to other immune base assays like latex agglutination and haem-agglutination assays. Moreover other advantages includes its simple operating and flexibility with cost effectiveness (Sato *et al.* 1996). For most of assays based on ICT only plasma or serum could be used as red blood cells interfere with the results interpretation because of color. However, in recent past new method has been developed and validated that one can use whole blood in these ICT-based assays (Shin *et al.*2001).To date, the only assay capable of HBsAg detection in whole blood, plasma or serum is the ICT hepatitis B sAg/eAg test. This can be attained in a single step without any blood pretreatment processes. Studies also revealed that the accuracy of the ICT based rapid tests is equally similar in fresh whole blood or sera and

for stored frozen sera as well (Lau *et al.* 2003). All these advantages make the ICT base tests more attractive in diagnosis field.

2.18 Non-invasive screening for Steatosis evaluation

Hepatic steatosis is characterized by lipid buildup in hepatocytes and is considered a simplest stage in NAFLD. Almost 30% of the general population is affected which demonstrated in 90% of the obese population in the United States. Progression to the non-alcoholic steato-hepatitis occur which results in inflammation of the hepatocytes. The gold standard method for the screening of steatosis is liver biopsy. However multiple risks are associated with this technique which may range from normal pain to life threatening bleeding. It may also include sampling error and therefore multiple non-invasive techniques have been proposed to increase the capacity for NAFLD diagnosis. These techniques include but not limited to computed tomography (CT), Ultrasonography (US), magnetic resonance imaging (MRI), spectroscopy and elastography.

2.18.1 Serological markers

Several different kind of serological markers are also studied to provide indication for hepatic steatosis. These include the ALT level, AST/platelet ratio index as discussed above (Fracanzani *et al.* 2008). CK18 fragments a stable proteolysis, have been reported with moderate accuracy for fibrosis. Different CK18 fragments correspond to hepatocellular death but sometime do not appear to be more precise (Joka *et al.* 2012).

2.18.2 APRI

Cirrhosis and fibrosis in HCV is significantly indicated by aspartate aminotransferase (AST)/platelet ratio index (APRI) (Wai *et al.* 2003). The APRI proposed formula for calculation is APRI = [(AST/ULN)/Platelet count] ×100, where upper normal limit is shown by ULN which is proposed to be 34 U/L in females and 36 U/L in males. This technique has advantages over the other non-invasive techniques. This is rapidly available blood test and simple to use. The role of APRI has been evaluated recently in HBV. Two different set of scales has been proposed for interpretation of APRI, where the initial scale is used to identify cirrhotic patients using APRI cut off score >2 while for

cirrhosis to roe out using cut off value <1 respectively. The clinically significant fibrosis is detected by second scale a cut-off value of APRI score >1.5 indicate the fibrosis where it is ruled out by score <0.5. A meta-analysis study published in 2012 demonstrated that APRI give areas under the receiver operating characteristic curves (AUROCs) significantly for cirrhosis and fibrosis of 0.75 and 0.79 respectively, including 9 studies (Jin *et al.* 2012). APRI with cut-off of 0.5 revealed significant fibrosis with specificity of 41% and 84% of sensitivity while 49% of sensitivity is characterized by cut off of 1.5. The cut-off range for cirrhosis is 1.0-1.5 with a specificity and sensitivity of 78% and 54% respectively while cut-off value of 2.0 showed 28 and 87%. All this aforementioned data presentation suggests that there is limited application of APRI in significance and identification of cirrhosis or fibrosis in HBV.

2.18.3 Ultrasonography (US)

In NAFLD patients with elevated hepatic enzymes, US is the pioneer frontier in diagnosing. This technique is considered as precise as live biopsy, with advantages for being a non-invasive technique with high reproducibility and low cost and no use of radiation (Singh *et al.* 2013). In the echotexture there is presence of homogenous hepatic normal tissue similar to the right cortex of the kidney and parenchyma of spleen. ON the US imaging the intracellular NAFLD fat droplets are characterized by higher echogenicity. Moreover, the hepatic steatosis may accompany hepatomegaly and blurring of hepatic vein vascular (Lee *et al.* 2014). 2-5 MHz probe with a convex structure is used for proper examination with US. Depending on the percentage of affected hepatocytes the liver steatosis can be graded from normal to sever using US. There are several limitation of US when detecting the mild level of fatty liver, or disadvantage is observed when used to distinguish steatosis for hepatic fibrosis (Saadeh *et al.* 2002). Studies demonstrated 60% sensitivity and specificity of about 80% for NAFLD detection through US. Despite of the fact that US is relatively easy to interpret and perform there are some limitations like lack of quantitative assessment in case of liver steatosis lower than 20%, difficult to detect (Iijima *et al.* 2007). There is also some inaccuracies reported in related to obese patients and result interference is operator dependent therefore additional research/studies are required to upgrade US for clinical investigations.

2.18.4 Computed tomography *(CT)*

An accurate and reliable visualization of the liver can be obtained by using CT a non-invasive technique. This could not only be used for diffuse but also the focal fatty infiltration can be studied and diagnosed accurately in parenchyma of liver (Dina *et al.* 2010). By measuring the spleen and liver attenuation differences in Hounsfield units the hepatic steatosis can be diagnosed (Piekarski *et al.* 1980). A decrease attenuation value of parenchyma is presented in non-enhanced CT images which correspond to hepatic steatosis. This is probably due to the hepatic attenuation and hepatic fat content inverse relation. The moderate to severe steatosis can be diagnosed by this method with almost reported 95-100% specificity. However, up to mark application of the CT in hepatic steatosis in NAFLD patients is limited of several different reasons like radiation exposure risk and high cost that make it difficult for patients to use in follow up. There also a decline in ability to detect steatosis as progression occurs with time and hence difficult sometime to diagnose complicated cases (Dina *et al.* 2010)

2.18.5 MRI and ^{1}H-Magnetic resonance spectroscopy

MRI and magnetic resonance spectroscopy both showed excellent accuracy in diagnosing steatosis (Permutt *et al.* 2012; Karlas *et al.* 2014). These techniques can be correlates and even reported superior when compared to other techniques like US, CT in term of specificity and sensitive. MRI however in patients with more severity, reported to be least effective in particularly for grading steatosis. However, the MRS showed good results. The other advantage of MRS is that this technique is operator independent which reduce the error (Lee *et al.* 2010). High cost and limited availability of these techniques are the disadvantages, although there is no exposure of the individuals to radiations. MRS is also considered a breathing free method so the results might have effected by breathing process as well.

2.18.6 Fibroscan

Transient elastography is used to measure stiffness in liver through acquisition of pulse echo ultrasound. The measurement is taken in kilopascals (kPa). This technique is

considered as non-invasive for the liver stiffness assessment in a volume approximately 100 times greater that obtained in liver biopsy which is invasion of the tissues. Taking in to consideration that portal hypertension and liver cirrhosis are highly associated to poor survival and it's important to categorizing the non-invasive procedures to accurately predict any clue for fibrosis and portal hypertension. FibroScan was initially used by Corpechot and its research colleagues to check the patients for biliary fibrosis with chronic cholestatic liver disease. Next they observed a very interesting correlation between the liver histological features, stage of fibrosis and liver stiffness on biopsy.

The significant of this technique was to accurately identify the fibrosis and 69 0ut of 95 patients (72.6%) were predicted accurately. A separate study conducted in Spain demonstrated 55 PBC patients that were evaluated for liver biopsy and transient elastography. Recently assessment carried out of the 45 PBC patients with liver fibrosis with FibroScan MR Spectroscopy, MRI and other serum marker analysis for fibrosis. All the results were plotted and compared with clinical and histological findings obtained after liver biopsy. Finally comparable results were demonstrated by the investigators between FibroScan and MRI with any accuracy of 80-83% in identifying the later stages of fibrosis i.e stage II. (Lindor and Levy 2012)

Material and Methods

3. Design and settings

This study was divided into two phases, in the early stage non-hospitalized individuals were screened for HBsAg to evaluate the prevalence. This cross-sectional study was carried out from August 2011 to August 2013. The first part of the study was focused on HBV prevalence and HBV co-infection with HDV and HCV. Later on we collected second group of patients (including Chronic HBV, steatosis and clearance or spontaneously recovered negative for HBsAg but positive for HBcAb and HBsAb) for genetic analysis and carried out in Khyber Pakhtunkhwa region during 2016 to 2017. Second part of study was focused on steatosis and chronic HBV persistence. The whole work was divided into three different categories, and each part was published separately. Hayatabad Medical Complex (HMC) was selected as a recruitment site of samples, which is one of the Provincial Sentinel Site for infectious disease surveillance. For second part of research work we collected Gastro OPD and Gastro wards patients of Hayatabad Medical Complex Peshawar. Most of routine work has been done at HMC Peshawar except from molecular biology work including PCR, Gel electrophoresis, genotyping and writing part of the work which was performed at Department of Microbiology QAU Islamabad and the Westmead Institute for Medical research.

3.1. Geography of the area assigned for study

Pakistan has four provinces, with two autonomous regions (Gilgit Baltistan and Azad Jammu and Kashmir) and Islamabad which is a capital territory. Khyber Pakhtunkhwa is situated in the north western constituency of the country beside the international border of Afghanistan. The South-East border is joined to Punjab, and the North-East border connected to Gilgit-Baltistan and the East border of KPK are merged with Azad Kashmir. KPK South border are connected to the Baluchistan province while its North-west borders are cognate with Afghanistan. Pashto speakers are predominant inhabitants in this region known as Pashtuns, while small numbers of other traditional group also existing in this area. Peshawar is the historical city and capital of this province. It is home to 17.9% of Pakistan's overall population. KPK comprise of a total of 34 districts.

3.2. Ethical approval for the study

For ethical acceptability of the study, permission was obtained from the Human Research Ethics Committees which is a registered Ethics committee at HMC Peshawar. All the mandatory standard of the Declaration of Helsinki were followed in this study.

3.3. Informed Consent from Patients

Signed Informed consent was obtained from each patient participating in the study. Patients were informed about the risk and discomfort of being participating in this study. Confidentiality of the data was strictly maintained.

3.4. Data collection and entry

A specially designed questionnaire was established for collection of patient data which was completed by a trained interviewer, containing information regarding age of the patient, sex, socioeconomic status, vaccination history, demographic, anthropometric data and various risk behavior for the study objective. After completion of patient proforma all the informations were entered into excel sheet for safe storage and preventing loss of the data.

3.5. Sample Collection:

Blood samples of the patients were obtained by trained and experienced phlebotomist using aseptic technique. Samples were collected from those who were fell within specific case selection criteria. A total of 3 to 5 ml of whole blood was taken in two tubes, gel tube (vacutainer) and EDTA from each patient. After centrifugation serum was separated and kept at -20°C for further testing. Standard operating procedures were strictly followed during each single step to reduce the errors.

3.6. Exclusion criteria

The selection criteria of subjects for the first part of the study was set to include those individuals, visiting outpatient department having no prior history of viral hepatitis were included in this study while previously vaccinated individuals against HBV were excluded. For the second part of the study patients were excluded certainly, if they were found being vaccinated against HBV in the past, evidence of co-infection with either HCV, human immunodeficiency (HIV) virus or hepatitis delta virus (HDV), or had evidence of other liver diseases. These patients announced no history of interminable liver illness and did not abuse alcohol (< 20 gm of alcohol every day).Only patients who were positive with both APRI and Ultrasound examination method were considered steatosis positive patients.

3.7. Evaluation of Hepatosteatosis

In current study for the purpose of steatosis evaluation in patients, US (Ultrasound) examination was used to measure the degree of hepatic steatosis. Ultrasound is additionally a good technique for steatosis evaluation with excellent specificity and sensitivity to detect moderate to sever steatosis level. For the purpose of steatosis grading different US features uses including contrast between the liver and the kidney, other feature are intrahepatic vessels appearance, liver parenchyma and diaphragm. Hepatic ultrasonography was performed for all the subjects by a trained sonologists. Hepatic steatosis is typically classified using B-mode images as none, mild, moderate, or severe

3.8. Laboratory assessment

Demography and clinical data were obtained at the time of blood collection including, sex, age, ethnicity, alcohol intake, socioeconomic status, vaccination history, symptoms and information related to various risk behaviors. Where hepatic steatosis was evaluated based on ultrasound and APRI. Sample of blood were collected in EDTA and Heparin vacutainer tubes to avoid clotting. The laboratory investigations were performed at main pathology department HMC some molecular tests were accomplished at the Department of Microbiology, Quaid-i-Azam University Islamabad. Genotyping of SNP for variants

identification was carried out at WIMR (University of Sydney Australia) supported by research funds of Higher Education Commission Pakistan. The lab investigations were carried out through different stages followed by SOPs.

3.8.1. Biochemical Test

The liver function tests conducted includes bilirubin, alkaline phosphatase (ALP) and serum ALT, by using Cobas C111 of Rosh and followed manufacturer's instructions and SOPs. Similarly, lipid profile test includes cholesterol and triglyceride was also performed on automated chemistry Cobas C111 (Roche, Switzerland).

3.8.2. Haematological analysis

EDTA tube sample was preceded for full blood count, including for platelets count. Sysmex KX21 haematology analyser of Japan was used to performed complete blood count and Platelets. Similarly the rest of the blood sample was stored for genomic DNA extraction.

3.8.3. AST Platelets Ratio Index

Although liver biopsy is gold standard for NASH diagnosis but, due to some limitations of liver biopsy, we used noninvasive method APRI to confirm steatosis in patients. So, all chronic HBV infected individuals were analysed by this method. The standard formula as discusses early, was used to measure the degree of steatosis and then all confirmed samples were further preceded for molecular analysis. The AST to platelet ratio index (APRI) was calculated according to the formula: (AST (IU/L)/upper normal limit) × 100/platelets (109/L).

According to the International Federation of Clinical Chemistry, the values for maximum normal AST is, 30 IU/L for women and 35 U/L for men. Similarly maximum normal range set for ALT is 42 IU/L in men and 26 IU/L in women.

3.8.4. ELISA

Samples were screened for the detection of HBs-Ag and positive samples were evaluated for anti-HBc-IgG, anti-HBc-IgM, anti-HBs-Ag, anti- HIV, anti-HDV and anti-HCV. For this purpose, commercially available ELISA kits (MBS-SRL, Milano, Italy) were used followed the instruction of the kits.

3.8.4.1. ELISA procedure for HBsAg and Anti-HBcore Detection:

- All the working reagents and other necessary chemicals were brought to room temperature and were keep at room temperature for one hour for further use. All the samples to be tested were also thawed before testing.
- The ELISA plate set up was made according to manufacture instruction. A1 well was left empty for blank. 50 µl of negative control was added to the next three wells (A2, A3, A4), similarly 50µl of positive controls was dispensed into the next two wells and finally a total of 50µl of patients sample added into their particular wells.
- Then 50µl of conjugate was added into each well excluding A1 blank. The contents of the plates were carefully mixed by gentle tapping the edge of the plates. Then we apply a plate lid to cover the plate safely and the reaction mixture was incubated at 37 °C for one hour in incubator.
- The plate cover was removed and plate wells were washed five time with washing solution in plate washer.
- In the next step we added both substrate A and B 50 µl, 50µl in quantity into each well including A1. The plate was cover to avoid light reaction with mixture of the substrate and incubated for 15 minutes at 37 °C in microplate incubator.
- 50µl of stopping solution was added into each well to stop further reaction. Then the micro plate was read at 450 nm.

Table 3.1. ELISA assay scheme for HBsAg and Anti-Hbcore

Reagents	Blank	Control	Samples
Controls	-	50 µl	-
Samples	-	-	50 µl
Conjugate	-	50 µl	50 µl
• Plate was incubated for 60 minutes at 37°. • Cover was removed and wells were washed 5 times with working washing solution.			
Substrate A	50 µl	50 µl	50 µl
Substrate B	50 µl	50 µl	50 µl
At 37°Cin microplate incubator all the samples were incubated for 10 minutes and the plates were covered by the lid of incubator to protected from light			
Stopping solution	50 µl	50 µl	50 µl
Blank was read at 620-630 nm for plate background measurement. While the control and samples were read at 450 nm.			

3.8.4.2. Procedure for Anti-HBs, Anti-HIV, Anti-HDV and Anti-HCV:

- All the reagents were brought to room temperature an hour before use. All the samples were thawed before testing.
- 100 µl of sample diluent was dispensed into each well except blank well. A total of 10 µlof negative control was added in next three wells (A2, A3, A4), similarly 10 µl positive control and 10 µl of test was included into their next respective wells.

- The reaction mixture was then mixed well by gentle tapping the edge of the plate. The plate was then sealed and incubated at 37°C for one hour in microplate incubator.
- After incubation the plate seal was removed and washed 5 times with washing solution inside the plate washer.
- 100 µl of conjugate was added into each well except blank well A1. Next the plate was again sealed with new plate sealer and incubated for 20 minutes in microplate incubator at 37°C.
- The second washing step, 5 times washing performed after completion of incubation by using working washing solution in automated plate washer.
- Then we added 50µl of substrate A and 50µl of substrate B, into all wells including A1. The plate was then subjected to 10 minutes incubation at 37°C in microplate incubator and the plate surface was covered with lid to protect from direct light.
- At the final step, the reaction was stopped by adding 50 µl of stopping solution into each well. Blank was read at 620-630 nm for plate background measurement. While the control and samples were read at 450 nm by microplate reader.

Table 3.2. ELISA assay scheme for Anti-HBs, Anti-HIV, Anti-HDV and Anti-HCV:

Reagents	**Blank**	**Control**	**Samples**
Sample diluents	-	-	100 ul
Controls	-	10 µl	-
Samples	-	-	10 µl
• Plates were covered and incubated for 30 min at 37°C • After proper incubation, then plate was 5 times washed with washing solution in microplate washer.			
Conjugate	-	100 µl	100 µl
• Plate was again sealed and incubated for 20 min at 37°C • Second washing was done in microplate washer, after completion of second incubation.			
Substrate A	50 µl	50 µl	50 µl
Substrate B	50 µl	50 µl	50 µl
After adding Substrates microplate was incubated for 10 minutes at 37°Cin microplate incubator. Plate was covered to protect from light.			
Stopping solution	50 µl	50 µl	50 µl
Blank was read at 620-630 nm for plate background measurement. While the control and samples were read at 450 nm.			

3.9. Molecular analysis

3.9.1. Extraction of DNA:

A total of 150µl of HBsAg positive serum was used to performed viral DNA extraction by using "Sacace" Biotechnologies extraction kit following manufacture established guidelines.

1. A total of 600 µl of prepared Buffer RAV1 (RAV1+RNA carrier) and 20µl of proteinase K was added to150ul of serum sample in a 2 ml micro centrifuge tube. The contents of tube were mixed by pulse vortexing for 20 seconds and the mixture was then incubated at 70 °C for 5 min.
2. Then 600 µl of absolute ethanol was also added and mixed by pulse –vertexing for 15 sec. A total of 700µl of the supernatent mixture was transferred to spin column (Ribo virus column), and was spined at 8000 x g for 60 second. The above step was repeated with the remaining mixture (new collection tube).
3. The filtrate along with tube was discarded and next the column was put in a new collection tube. Wash bufferof 500µl (RAW buffer) was dispensed to the column and then spined at 8000 x g (8000rpm) for 1 minute.
4. After spinning, the collection tube was again discarded and the column was place in new collection tube. Secondary wash buffer of 600 µl RAV3 was dispensed to the column and then spined at 8000 x g for 1 minute.
5. After spinning, the filtrate was discarded and the empty column was placed in 2 ml tube for collection of sample mixture. A total of 200 µl of RAV3 buffer was transferred to the column and then spun at 11000 x g for five minutes to remove ethanolic buffer RAV3 completely.
6. The column with open cap was incubated at 70 °C for 1 minute to remove any remaining traces of ethanol.
7. Then column was shifted in a new, sterile 2.0 ml centrifuge tube. Finally, 50µl RE buffer (preheated to 70°C) was transferred and then incubated for 1-2 minutes. Then centrifugation was done for 60 seconds at 11000 x g to collect the DNA. The DNA was Stored at -70 °C.

3.9.2. Real-time PCR quantification of HBV using SmartCycler II

The DNA of HBV was extracted from patient serum by using SACACE Biotechnologies S.r.l, Italy commercially available kits, and PCR was then accomplished on a SmartCycler II apparatus (Cepheid make) by means of a SACACE HBV monitor kit (Germany). The target DNA amplification was accomplished:by initial denaturation at 95°C for 15 minutes, followed by 35 cycles of denaturation at 95 °C for 20 sec, while annealing and extension was carried out at 60 °C for 40 sec.The fluorescence was estimated at the annealing stage of each cycle. The quantitative analysis of each sample subjected to be tested was done by using the value of internal control co-effient and the fluorescent value of both sample and internal control.

3.9.3. Genomic DNA extraction and purification

1. A total of 400 µl of Lysate Solution and 20 µl of Proteinase K Solution were mixed with each 200 µl of whole blood sample. The mixture was then vortex and pipet repeatedly to obtain a good suspension.
2. The suspension was incubated at 56 °C for 10 minutes and cell lysis was done by vortexting or thermomixer, or by using shaker water bath.
3. Similarly a total of 200 µl of ethanol which was 96-100% was added to the suspension. The suspension was then mixed properly by vortexing.
4. The resulting lysate mixture was relocated to a DNA purification column and each column was serially put into a collection tube. The column was spun at 6000 x g for 1 min, the collected filtrate was removed from collection tube. The purification column of genomic DNA was positioned in a new 2 ml tube for further washing.
5. After adding 500 µl of wash buffer I (already added ethanol) into a column, then the column was centrifuge at 8000 xg for 1 min. The filtrate was removed from collection tube and the purification column was again placed into the collection tube.
6. Similarly, 500 µl of wash buffer II was next added to the DNA purification column. Spinning the column for 3 min at maximum speed (≥12000 x g). An additional I min high speed centrifugation was done to remove any suspended liquid in column. The

collection tube was discarded and the purification column was transferred into a sterile 1.5 ml eppendrof tube.

7. Finally, 200 µl of elution buffer was added to the center of the purification column film to elute genomic DNA. The column was incubated for 2 min at room temperature and spinnedfor 1 min at 8000 x g.
8. The purification column was discarded and the eluted purified DNA was stored at -20 °C.

3.10. Gel Electrophoresis

Agarose gel electrophoresis was done to confirm the presence of purified DNA. Agarose gel (1%) was prepared by dissolving 1g of agarose in 100 ml of 1X TBE buffer by heating in oven for 1 min. The resulting solution was poured into gel casting tray, containing comb for the formation of wells and allowed to cool down. The gel was solidified within 10 min and later on the gel tray was placed in the BioRad gel tank containing 1X TBE buffer. The level of running buffer in the gel tank was adjusted in accordance to cover the surface of the gel. The sample subjected to be loaded in the gel wells were prepared by mixing 5 µl of DNA sample with 1 µl of 6X loading dye (Fermentas). About 3 µl DNA marker (Thermo Scientific) was loaded as marker for determining the size of DNA. Samples were loaded into their respective wells, the lid was placed on the gel tank and gel was run for 30 min at 90V. Afterwards, the gel was observed on ultra-violet (UV) illuminator for the presence of DNA bands. After confirmation these DNA samples were transported to the Westmead Institute of Medical Sciences Sydney Australia for further analysis.

3.11. DNA Quantitation

Purified DNA was quantified using Thermo Scientific Nanodrop (Nanodrop 1000 spectrophotometer). Quantity of the measured DNA was recorded in terms of ng/µl.

3.12. Candidate gene selection

To analyze the single nucleotide polymorphism (SNP) and their outcome of chronic HBV infection, hepatosteatosis and viral clearance, five sets of genes in the immune system

plethora were targeted. The targeted gene located on different chromosomes. The genes studied include *IFNL3* rs12979860, *STAT4* rs7574865, *PNPLA3* rs738409, *MBOAT7* rs641738 and *TM6SF2* rs58542926.

Table. 3.3. Probe used for the SNP analysis of desired genes.

SNPs	**Probe**
***STAT4* rs7574865**	
TATGAAAAGTTGGTGACCAAAATGT**[G/T]**AATAGTGGTTATCTTATTTCAGT	
***IFNL3* rs12979860**	
TGAACCAGGGAGCTCCCCGAAGGCG**[C/T]**GAACCAGGGTTGAATTGCACTG	
***PNPLA3* rs738409**	
AGGCCTTGGTATGTTCCTGCTTCAT**[C/G]**CCCTTCTACAGTGGCCTTATCCCC	
***TM6SF2* rs58542926**	
GTGAGGAAGAAGGCAGGCCTGATCT**[C/T]**GGAGCTGTATTTGCCTTCCATGT	
***MBOAT7* rs641738**	
TCTGGCCTCCCGGGGGGCCAGCCAC**[C/T]**CCCTAGAGGAGCCCCAGGCTTCG	

Every allelic discernment probe set contains two probes, one probe for allele 1 and one probe for allele 2.

Table. 3.4. PCR Reaction Mix

PCR Mixture for each sample	
TaqMan Master Mix	N x 7.5 ul
DNA	N x 1 ul
Probe	N x 0.3 ul
Nuclease free water	N x 6.2 µl
Total volume	N x 15.0µl

3.13. Genotyping

Genotyping for *STAT4*(HGNC: 11365) rs7574865,*IFNL3* (HGNC: 18365) rs12979860,*MBOAT7* (HGNC: 15505)rs641738, *PNPLA3* (HGNC: 18590) rs738409and *TM6SF2* (HGNC:11861)rs58542926 was carried out utilizing the TaqMan SNP genotyping allelic discernment technique (Applied Biosystems, Foster City, CA, USA). Genotyping was blinded to clinical variable. The reaction condition and temperature were set up as fallowing: Initial denaturation at 95˚C for 10 minutes, followed by 40 cycles at 95˚C for 15 seconds and 60˚C for 1 minute and finally for 30 sec at 60˚C.

3.14. Statistical Analysis

For the prevalence part the data were recorded in MS excel and standard descriptive analysis was carried out by using MS excel and graph pad prism software version 5. The distributions of various attributes of infections were explored across different demographic variables. Distributions were separately established for the male and female samples. Co-infections, clinical details, and risk factors were analyzed accordingly. Chi-test was performed to check the significance of distributions among different variables.

For the second part of the study the data are represented as mean and standard deviation, median and range or number and proportion, as appropriate. Hardy Weinberg equilibrium tests for STAT4 rs7574865 and IFNL3 rs12979860 were performed using the chi-squared test, and the difference in the genotype distributions between the different groups was compared using Fishers exact test or the Cochran Armitage test for trend. The Students t-test or a nonparametric test, i.e. the Wilcoxon Mann Whitney U test or the Kruskal Wallis test, was used to compare quantitative data, wherever appropriate. All tests were two-tailed, and p values < 0.05 were considered statistically significant. For rs7574865, a recessive model (GG versus GT/TT) was implemented, as described previously, the model was an estimate of the odds ratio (OR) for disease risk in an individual with no A alleles (genotype a/a) compared with that in an individual with at least 1 A allele (genotype A/A or a/A). For IFNL3 rs12979860, a dominant model (CC vs CT/TT) was implemented, as reported previously [1820]; this model was an estimate of the OR for disease risk in an individual with two A alleles (genotype A/A) compared with that in an individual with one or two A alleles (genotype a/A or a/a).

The steatosis part data are also shown as mean and standard deviation (SD), median, range or number, and proportion, as appropriate. The frequency of PNPLA3 rs738409, MBOAT7 rs641738, and TM6SF2 rs58542926 genotypes was compared between different groups using Fisher's exact test. The Cochran-Armitage test was used for the assessment of trends. The student's t-test or non-parametric Wilcoxon-Mann- Whitney U-test or Kruskal-Wallis test was used to compare quantitative data, as appropriate. All tests were two-tailed and P values of < 0.05 were considered significant. Hardy-Weinberg equilibrium tests of PNPLA3 rs738409, TM6SF2 rs58542926, and MBOAT rs641738 were performed using the chi-square test. The HBV DNA levels were log-transformed before modeling.

The logistic regression model was used and the results are expressed as OR and 95% CI. Statistical investigations were performed using statistical software package SPSS for Windows, version 21 (SPSS, Chicago, IL).

4. Results

A. First part (Prevalence of HBV, HDV and HCV co-infection and related factors)

4.1. Sample characteristics and prevalence of HBV

A total of 946 non-hospitalized patients were screened in first phase, out of 212 were HBsAg positive. All positive patients were further tested for HCV and HDV co-infection. Out of total 946 cases53% ($n = 501$) were males and 47% ($n = 445$) females. Overall the prevalence rate of HBsAg was 22.41% (prevalence: 224.10/1 000; *CI*: 0.197 5 ± 0.250 7, shown in Table 4.1). Relatively higher prevalence of HBV infection was observed in the male patients (prevalence: 227.54/1 000) in comparison to the female patients, prevalence: 220.22/1 000; OR: 1.04 (Table 4.1, Table 1.2). However, differences between the distributions of gender specific samples were not significant statistically ($P > 0.05$). To estimate the prevalence of HBV infection among different age groups, data were divided into six age categories. The prevalence of HBV infection was highest among younger age groups (Table 4.1). Although, a declining trend of HBV prevalence was evident between different age-intervals, differences in overall distribution of HBV infection across different age-groups were not significant statistically ($\chi^2 = 4.57$, $df = 5$; $P = 0.47$). No significant differences were evident when infected individuals were cross tabulated in different age categories against gender (shown in Table 4.2).

Table 4.1. Prevalence of HBV and co-infection with other hepatitis viruses in scrutinized population

Prevalence of hepatitis		Infected	Un-infected	Total	Prevalence/ 1000	Proportion	95% CI	Odd ratios*
Gender-wise Age-categories (Years)	HBV	212	734	946	224.1	0.224 1	0.197 5 ± 0.2507	16.7882
	HDV	23	923	946	24.31	0.024 3	0.014 5 ± 0.0341	1.4484
	HCV	16	930	946	16.91	0.016 9	0.008 7 ± 0.0251	Reference
	HBV + HCV/HDV	36	734	770	46.75	0.046 8	0.031 8 ± 0.0617	2.8508
	HBV (male)	114	387	501	227.54	0.227 5	0.190 8 ± 0.2643	1.043
	HBV (female)	98	347	445	220.22	0.220 2	0.181 7 ± 0.2587	Reference
	Up to 9	19	57	76	250	0.250 0	0.152 6 ± 0.3474	1.5185
	10–19	62	189	251	247.01	0.247 0	0.193 7 ± 0.3004	1.4944
	20–29	65	202	267	243.45	0.243 4	0.192 0 ± 0.2949	1.4658
	30–39	32	143	175	182.86	0.182 9	0.125 6 ± 0.2401	1.0194
	40–49	18	82	100	180	0.180 0	0.104 7 ± 0.2553	Reference
	50 and above	16	61	77	207.79	0.207 8	0.117 2 ± 0.2984	1.1949

4.2. Prevalence of co-infection with HDV and HCV

The prevalence of co-infection, *i.e.*, HBV with either HCV or HDV was witnessed in 36 patients (Table 4.2). Among the co-infected patients, 19 were males and 17 females. Overall, 10.85% (23/212) of the samples were reactive against HDV and 7.55% (16/212) against HCV. Out of all HDV infected patients, 43.48% (10/23) were males while 56.52% (13/23) were females ($\chi^2 = 0.851$; $df = 1$; $P = 0.356$) (Table 2). Unlike HDV infected individuals, higher percentage of HCV positive individuals 62.5% (10/16) were males in comparison to 37.5% (6/16) females($\chi^2 = 0.595$; $df = 1$; $P = 0.441$; not significant).

Table 4.2. Gender differentials in the distribution of infections and age-specific samples.

Infection		Male	Female	Total type/variable	
Hepatitis B	HBV	114	98	212	
	Non-HBV	387	347	734	$c^2 = 0.073$, $df = 1$,
	Total	501	445	946	$P = 0.788$, NS
Hepatitis D	HDV	10	13	23	$c^2 = 0.851$, $df = 1$,
	Non-HDV	491	432	923	$P = 0.356$, NS
Hepatitis C	HCV	10	6	16	$c^2 = 0.595$, $df = 1$,
	Non-HCV	491	439	930	$P = 0.441$, NS
Co-infection	HBV + HCV/HDV	19	17	36	
	HBV only	95	81	176	$c^2 = 0.089$, $df = 2$,
	Un-infected	387	347	734	$P = 0.956$, NS
HBV in age-categories (years)	Up to 9	12	7	19	
	10–19	28	34	62	
	20–29	39	26	65	
	30–39	15	17	32	
	40–49	9	9	18	$c^2 = 5.696$, $df = 5$,
	50 and above	11	5	16	$P = 0.337$, NS
	Total	501	445	946	

4.3. Detection of anti-HBC total antibodies and viral DNA

Among the 212 HBsAg-positive patients, anti-HBc-total was detected in 86.79%. Anti-HBc-IgM was detected in 25% of the patients out of which four (7.53%) were positive for anti-HDV and three (5.66%) for anti-HCV (Table 4.3). Viral DNA was detected in 64.13% ($n = 136$) of the HBsAg-positive patients and 11.51% ($n = 16$) of these were co-infected with HDV. Similarly, 5.14% ($n = 7$) of these patients were co-infected with HCV. Majority of the patients' positive for HBV DNA were young males. Among co-infected (with either HDV or HCV), anti-HBc was detected in 9.24% ($n = 17$) and 6.6% ($n = 14$) of the patients, respectively (Table 4.3).

Table 4.3 Detection of anti-HBc-IgM and anti-HBcIgG and viral DNA among HbsAg positive patients

Type of infection		No. of subjects	No. of HBc-IgM	No. of HBc-IgG	No. of DNA detected
Infection type	HBV + ve (total)	212	53	184	136
	HBV + ve (male)	114	37	104	72
	HBV + ve (female)	98	16	80	64
	HDV	23	4	17	16
	HCV	16	3	14	7
	HBV + HCV/HDV	36	7	31	22
	Only HBV	176	46	153	114
Age categories (years)	Up to 9	19	4	19	12
	10–19	62	20	53	38
	20–29	65	13	55	45
	30–39	32	10	27	19
	40–49	18	3	17	15
	50 and above	16	3	13	7

4.4. Symptoms and associated risk factors

Different clinical symptoms were observed among infected patients, including abdominal discomfort, fatigue, anorexia, malaise, fever, dark urine, jaundice, and splenomegaly (Table 4). Most of the symptoms were significantly associated with infections ($\chi^2 = 103.9$; $df = 7$; $P < 0.000\ 1$). Abdominal discomfort was one of the most frequently reported symptoms associated with HBV infection (79%) that was followed by fatigue (73%), anorexia (66%) and malaise (62%). Similarly, fever, dark urine and jaundice were higher among HBV infected patients (Table 4.4). Different risk factors were found to be associated with the infections. Particularly, the association of certain factors like injection, intravenous infusion, hospitalization, dentist visit, and surgery, were considerably higher among the infected groups of patients (Table 4.5). All other enlisted risk factors showed significant association with the infections ($\chi^2 = 95.23$; $df = 11$; $P < 0.000\ 1$).

Table 4.4. Clinical symptoms and complications associated with hepatitis infection among the scrutinized patients

Clinical symptoms*	Type of infection					
	HBV (n = 212)	HDV (n = 23)	HCV (n = 16)	HBV + HCV/HDV (n = 36)	Un-infected (n = 734)	Total (n = 946)
Abdominal discomfort	79.25	78.26	81.25	80.56	22.48	333
Fatigue	72.64	56.52	81.25	66.67	11.72	240
Anorexia	66.04	65.22	62.5	66.67	21.66	299
Malaise	61.79	65.22	68.75	66.67	11.44	215
Fever	53.77	47.83	50	50	22.75	281
Dark urine	44.34	43.48	31.25	38.89	12.26	184
Jaundice	26.42	26.09	37.5	30.56	11.85	143
Splenomegaly	0.94	0	0	0	8.58	65

$\chi^2 = 103.9$, $df = 7$, $P < 0.000\ 1$*

*: Distributed in the descending order of prevalence and shown as percent occurrence; **: Statistically highly significant; *Chi*-test was carried out through the values in HBV and un-infected subjects.

Table 4.5. Risk factors associated with hepatitis infections among the scrutinized patients

Clinical symptoms*	Prevalent hepatitis types and co-infection				Un-infected	Total
	HBV (n = 212)	HDV (n = 23)	HCV (n = 16)	HBV + HCV/HDV (n = 36)	(n = 734)	(n = 946)
Injection	87.74	73.91	81.25	80.56	37.19	459
Intravenous infusion	83.96	86.96	87.5	88.89	10.76	257
Hospitalization	46.7	47.83	50	50	20.84	252
Skin piercing	43.87	47.83	50	47.22	35.83	356
Visit to barber (male)	69.3	40	70	57.89	47.7	318
Dentist visit	39.62	47.83	37.5	44.44	6.13	129
Surgery	25.47	13.04	31.25	19.44	12.4	145
Blood transfusion	8.02	13.04	12.5	8.33	6.13	62
Contact with jaundice person	6.6	4.35	12.5	8.33	10.22	89
Tattoos/acupuncture	5.19	13.04	0	5.56	6.13	56
Visit to beauty parlor (female)	6.12	7.69	0	11.76	5.84	32
Illegal injection use	2.36	4.35	6.25	5.56	1.5	16

$\chi^2 = 95.23$, $df = 11$, $P < 0.000\ 1$**

: Distributed in the descending order of prevalence and shown as percent occurrence; **: Statistically highly significant; *Chi*-test was carried out through the values in HBV and un-infected subjects.

B. Second part (Chronic HBV persistence and Steatosis)

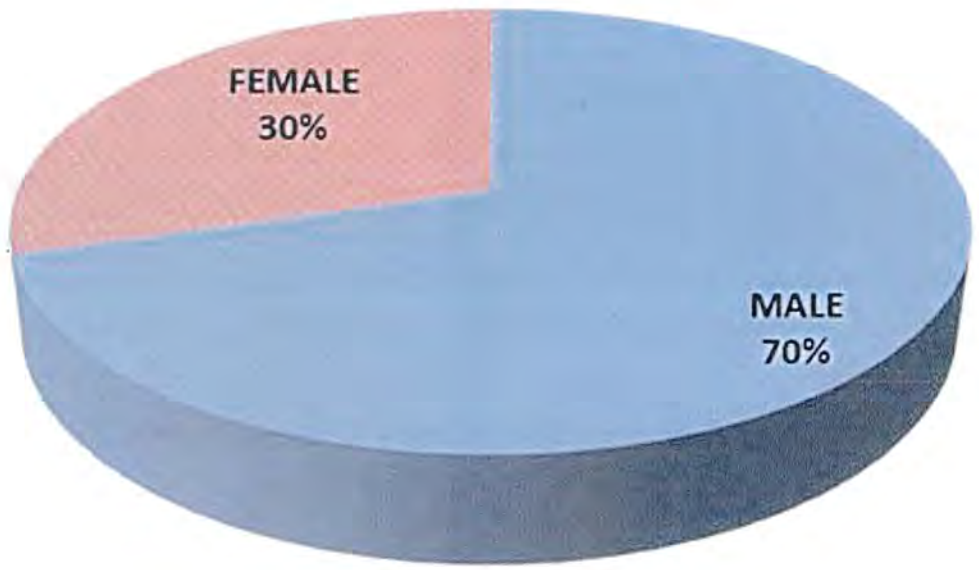
A total of 354 participants were included in this second phase of the study. For ease of understanding and to portray clear picture of the data we further break our data into two subgroups. Subgroup B1 including participants with evidence of clearance of HBV versus chronic HBV patients. While subgroup B2 including patients with steatosis versus those without steatosis chronic HBV patients.

B1. Group with evidence of clearance of HBV versus chronic HBV patients

4.5.1. Clinical and demographic analysis of the studied participants

Among these total 297 individuals, 57 were clearance cohort (spontaneously recovered patients)and 240 were chronic HBV patients. HBV patients were confirmed by 3rd generation ELISA. Gender based differentiation of the analysed HBV patients witnessed that n=169 (70%) of the participants were males whereas, n=71(30%) of the analysed patients were females (Figure 4.1).

Figure 4.1 Chart representing HBV prevalence among male and female patients



4.5.2. Regional Prevalence of HBV infected patients

Next we determine the distribution of the patients in different regional areas. A total of n=124 (52%) patients were found in Rural region, while 116 (48%) patients were found in Urban localities (Figure 4.2). (p=0.142, statistically not significant)

4.5.3.1. Prevalence of viral DNA in in HBV positive patients

Hepatitis B viral DNA detection was carried out for all 240 positive patients, and it was found that viral DNA was detected in n=154 (64%) of the total patients, while 86 (36%) of the patients were negative for HBV DNA presence (Figure 4.3). We also analysed the spontaneously recovered control individuals for HBV viral DNA, but all the samples were found negative.

Figure 4.2.Chart representing prevalence of HBV in rural and urban zones of KPK

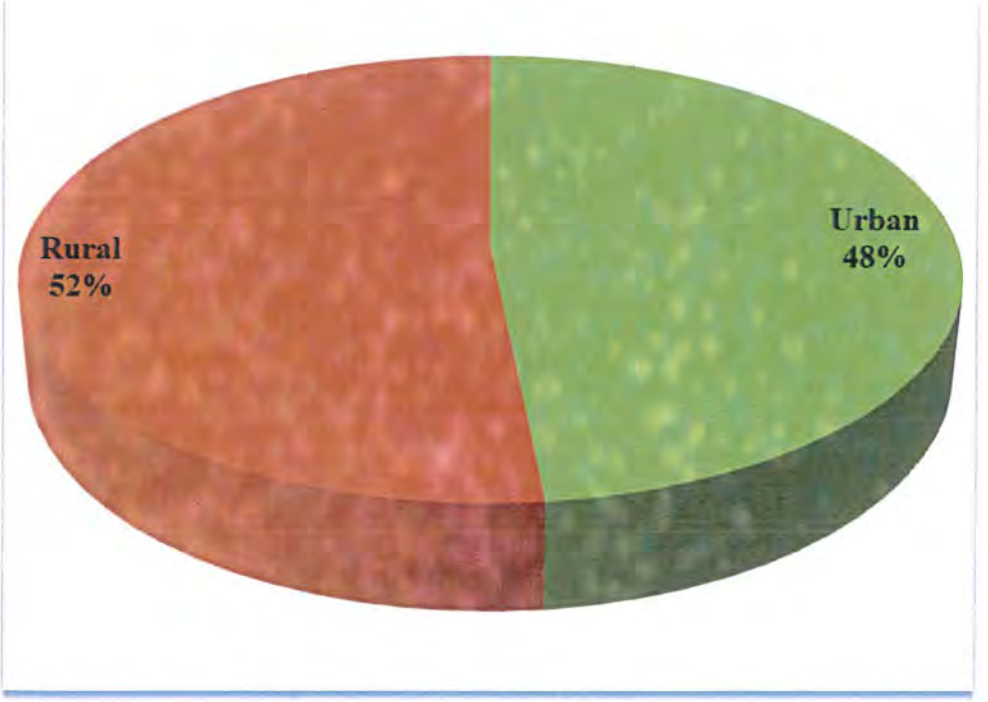
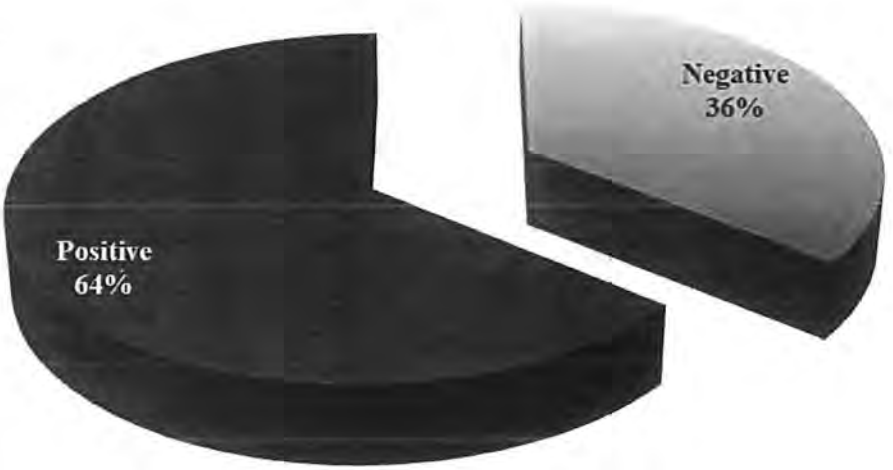


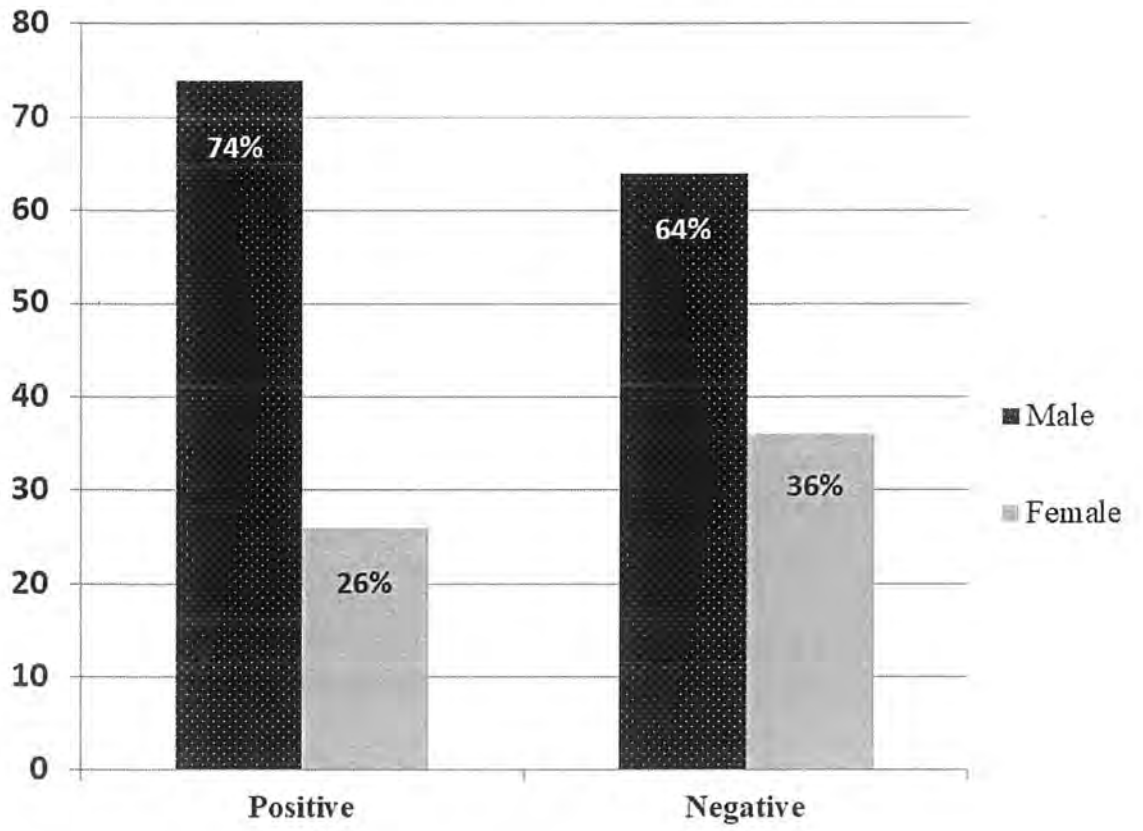
Figure 4.3. Prevalence of HBV DNA in Hepatitis B positive patients



4.5.3.2. Prevalence of viral DNA in HBV positive male and female patients

Gender based viral DNA distribution revealed that Hepatitis B viral DNA was present in n=114 (74%) of the studied males individuals while n=55 (64%) of the male patients were negative for viral DNA. Similarly, viral DNA distribution in females was n=40 (26%), while n=31 (36%) of the female patients were negative for HBV DNA (Figure 4.4). (p=0.107, statistically not significant)

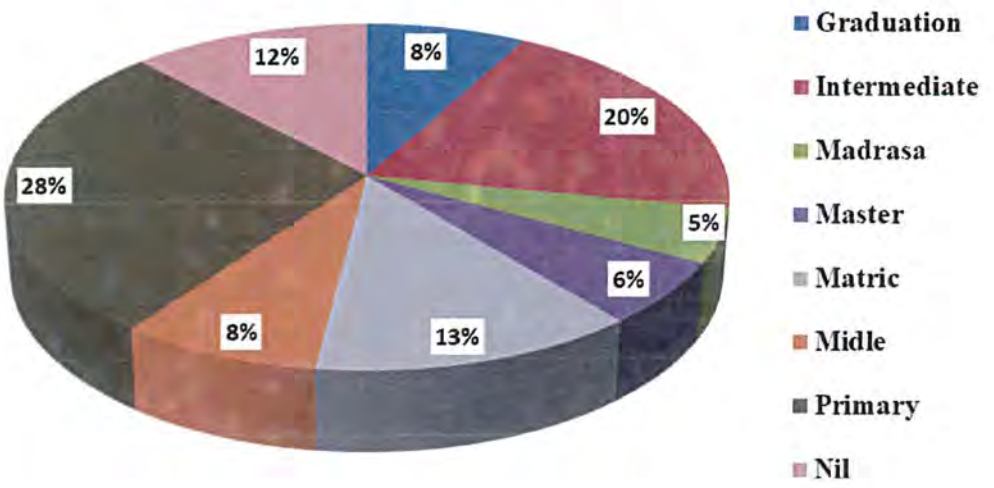
Figure 4.4. Gender wise prevalence of HBV DNA in Hepatitis B cohort



4.5.4. Literacy rates in studied participants.

Literacy rates in analyzed participants revealed that most of the participants had primary, n=67(28%) followed by intermediate and matric levels of education having rates of n=37(20%) and n=32(13%) respectively. Most of the participants are not highly qualified and it can be concluded that the individuals have less knowledge and, awareness about communicable disease. The literacy data is shown in (Figure no. 4.5).

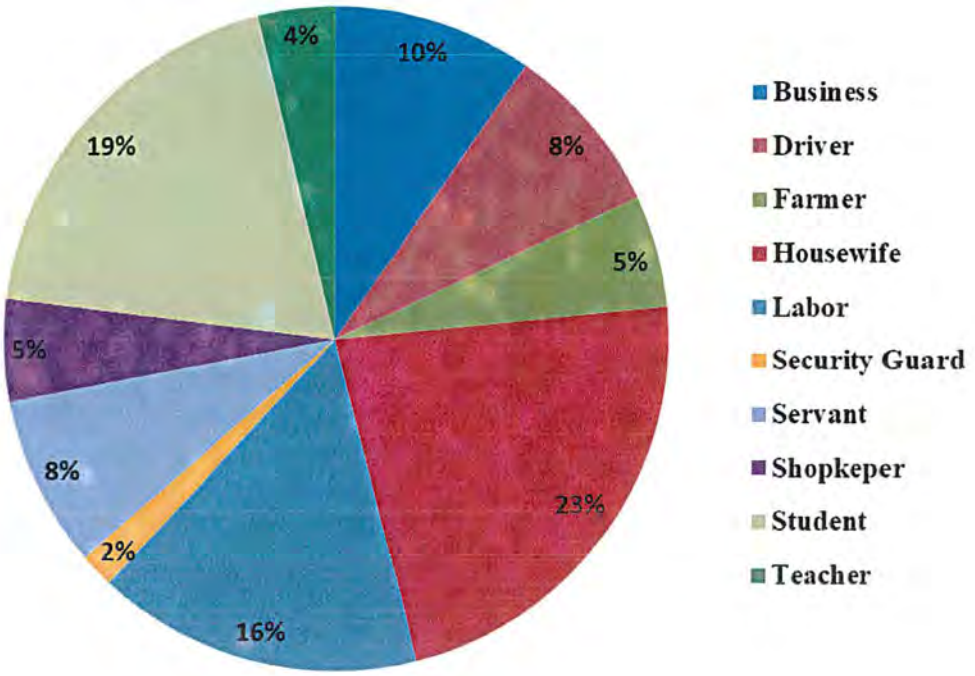
Figure 4.5.Chart representing literacy rate among studied participants



4.5.5. Occupation information of the studied participants

Data regarding the occupation of the participants was also obtained and it was found that the occupation was dominated by housewives, n=54(23%) belonging to female patients. Young students and laborers were the second most prevalent occupation in analyzed patients having rates of n=46(19%) and n=38(16%) respectively. Data for the rest of the occupations is shown in (Figure no. 4.6).

Figure 4.6. Chart representing occupation of studied participants



4.5.6.1. Distribution of age groups among studied participants

Age intervals for the studied participants revealed that majority of the participants belonged to age group 20-29 having prevalence rates of n=89(37%) respectively similarly the age group 10-19 also had second highest rates of n=51(21%) followed by age group 30-39, n=44(18%). Age groups up to 9 years had a lowest prevalence rate of n=5(2%). While the age group of 40-49 and 50 and above had also low prevalence rates of n=28(12%) and n=23(10%) respectively (Figure 4.7).

4.5.6.2. Age based distribution of male and female participants

The highest prevalence of male patients fell in the age group of 20-29 years n=77 (37%), followed by age group of 10-19 and 30-39 years which were n=56 (27%) and n=37

(18%) respectively. Similarly, the highest frequency of females was measured in age group of 20-29 which was n=30 (35%) and followed by age group 30-39 n=16 (18%) and 40-49 which was n=16 (18%). Although the lowest frequency of male and female was detected in age group of up to 9 years, which were n=3 (1%) and n=2 (2%) respectively. (p=0.101, statistically not significant) (Figure 4.8).

Figure 4.7. Prevalence of HBsAg among the study participant by age groups

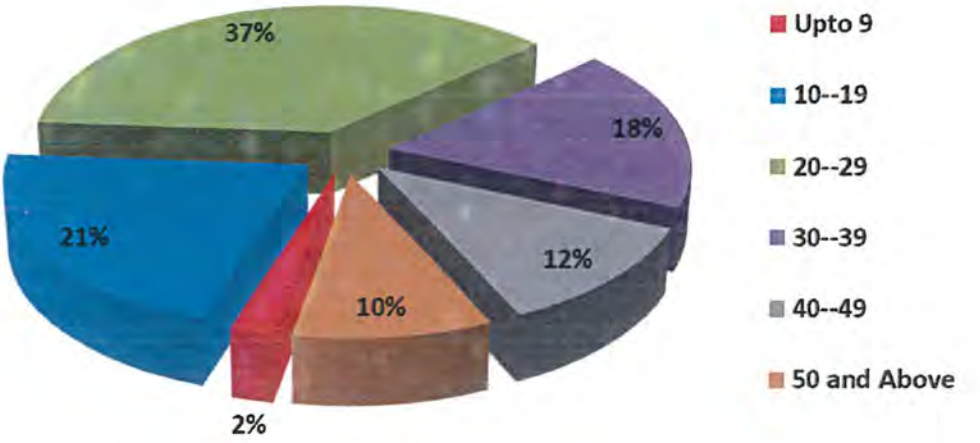
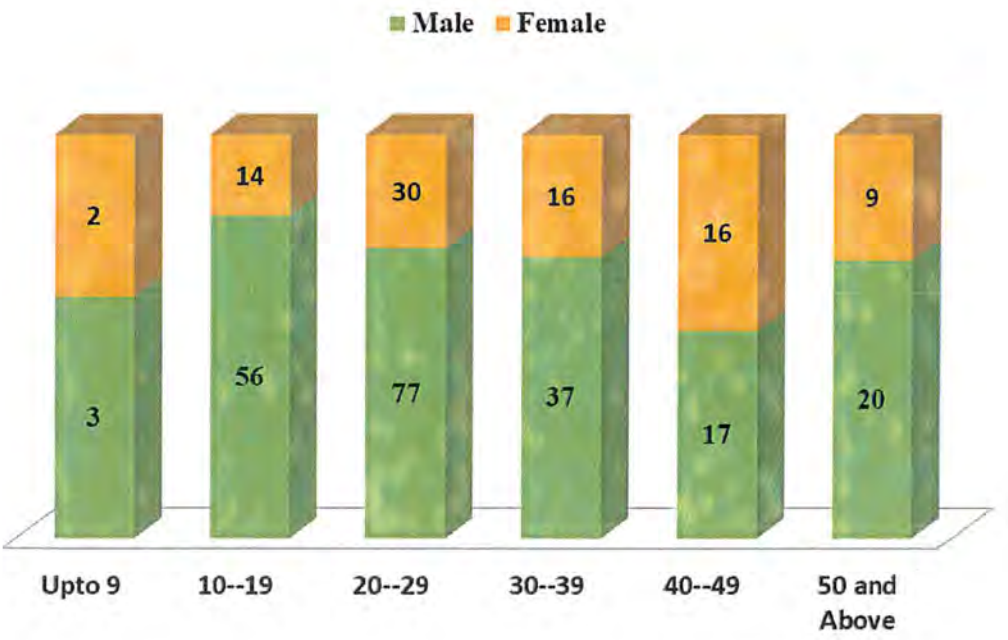


Figure 4.8. Age based distribution of male and female participants



4.5.7. Demography of the studied participants

Demographic analysis for all participants revealed that most of the participants belonged to Khyber Pakhtunkhwa provincial capital Peshawar region n=109 (37%), followed Khyber Agency and Charsadda n=61 (21%) and n=27 (9%) respectively. Participants from Batkhela and Lakki Marwat were lowest in number which were n=2 (1%) each. Participants from the other region of the county are shown in chart (Figure 4.9).

4.5.8. Source of water consumed by studied participants

Water source for drinking and other utility is categorized into different group. The highest percentage of people used Municipal water n=155(65%) for drinking. Then followed by second highest number of individuals n=78(32%) who utilized Well water. Low percentage of Participants consumed Spring/River water n=4(2%) and Surface water n=3(1%) for drinking purposes (Figure 4.10). Although HBV is not spread through

water, but we just analysed this variable to detect any relevance among infected individuals.

Fig 4.9. Demography of analysed participants

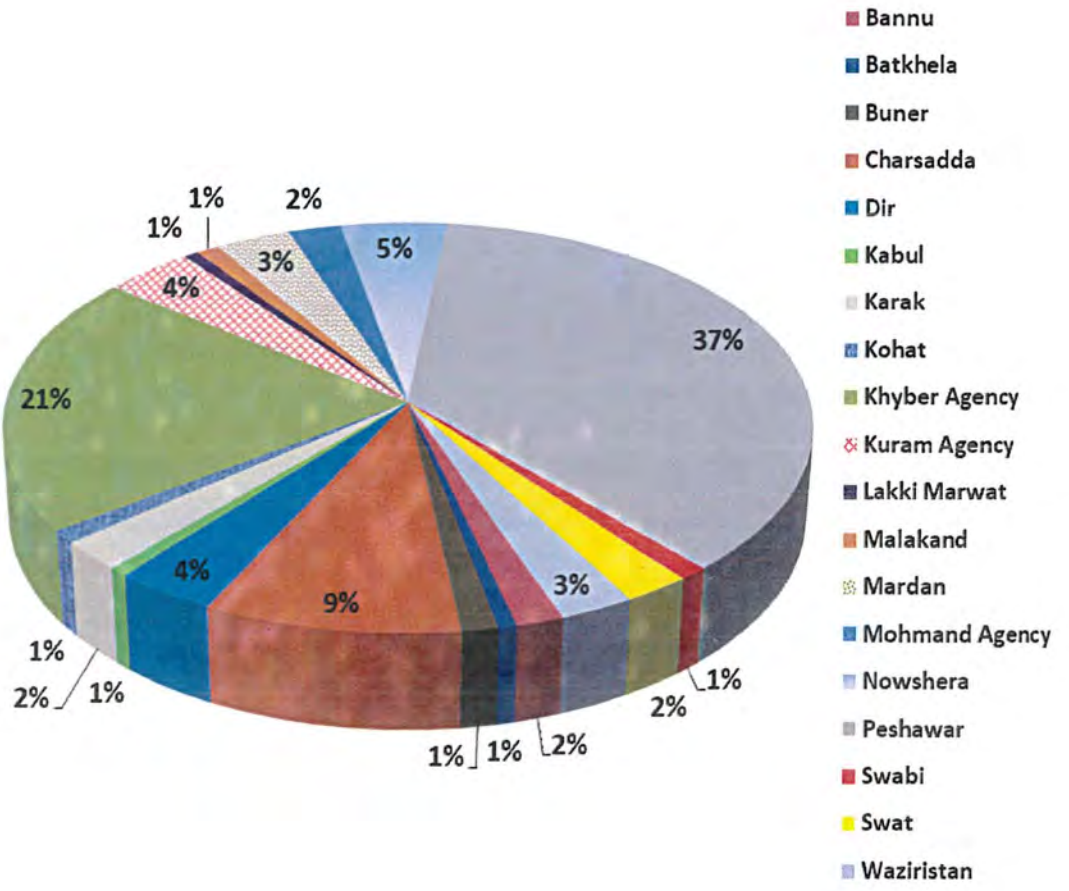
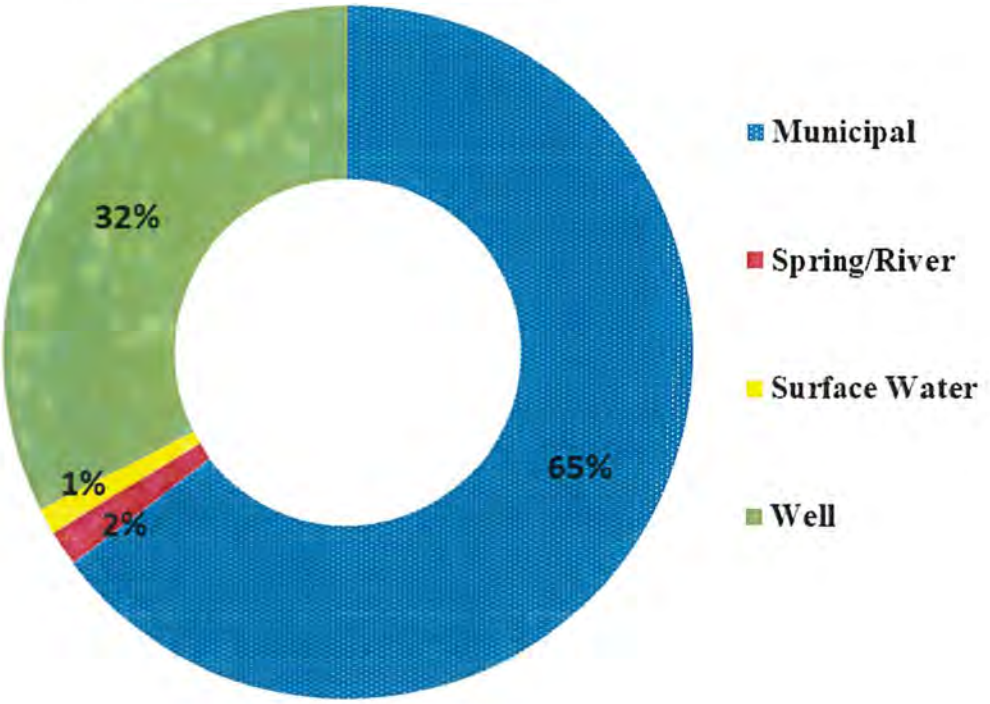


Fig 4.10. Source of water utilized by studied participants



4.5.9.1. Prevalence of signs and symptoms in clearance and hepatitis B cohort

Symptoms observed in clearance control and chronic HBV individuals revealed that majority of the symptoms were absent in clearance cohort while most prevalent symptom detected in HBV infected patients were abdominal discomfort, fatigue, anorexia and malaise respectively having the rates of n=190(79%), n=175(73%), n=156(65%), and n=148(62%). While Prevalence rates for the other sign and symptoms observed in this study like fever, jaundice, dark urine and hepatosplenomegaly are shown in the (Figure no. 4.11). (p=0.0001, statistically significant)

4.5.9.2. Gender based distribution of signs and symptoms in males and females

Symptoms differentiation in male and female participants revealed that abdominal discomfort was the most prevalent symptom in males as compared to females, n=140(67%) vs n=53(61%). The other significant sign found to be varied was hepatomegaly as males had the prevalence rates of n=27(13%) as compared to females

n=17(19%) respectively. Other studied symptoms like dark urine, fever Splenomegaly and rest of them were almost equally distributed among male and female individuals as shown in (Figure no. 4.12). Chi square distribution showed probability value of 0.218 and hence statistically not significant.

Figure 4.11. Signs and Symptoms analysis in clearance and hepatitis B cohort

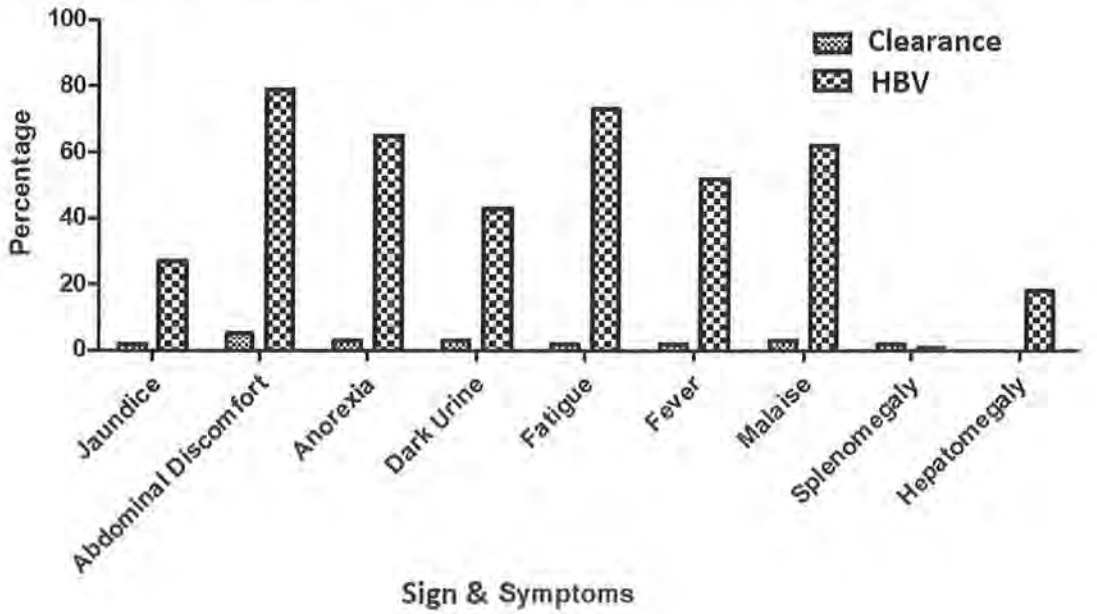
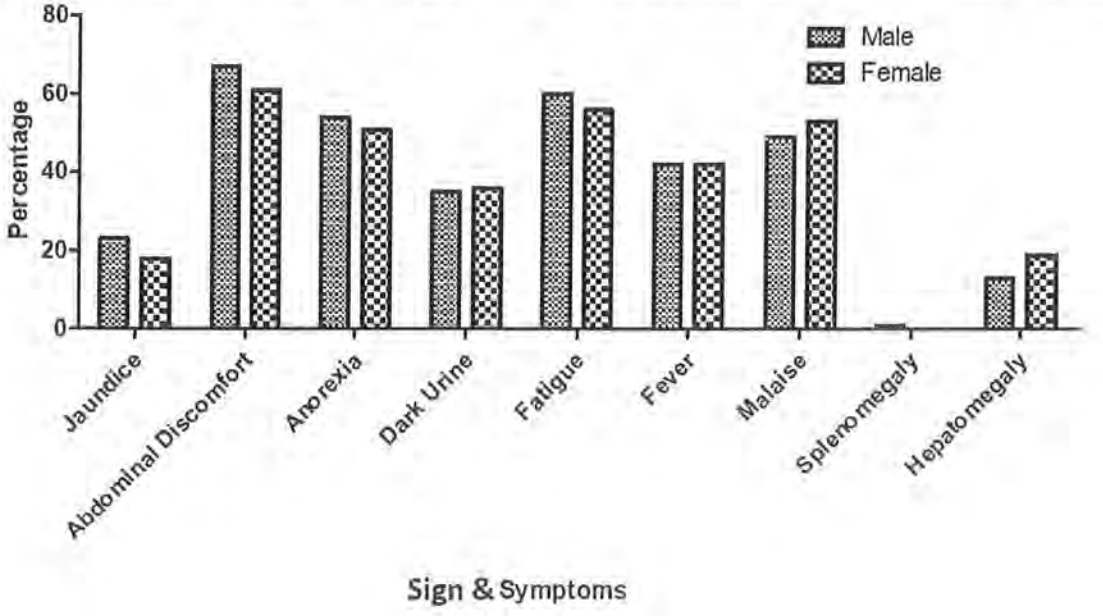


Figure 4.12. Analysed Signs and symptoms in males and females



4.5.10.1. Risk factors associated with HBV infection in analysed patients

Risk factors analysis was performed in both clearance and HBV cohort, but the rate and degree of associated risk factors were lower among clearance individuals as compared to hepatitis B infected patients as showed in (Figure no.4.13). The most predominant distributed risk factor among HBV patients was injection use n=210(87%) followed by intravenous infusions, n=200(83%). Barber visits and contact (close) with jaundice person were also the most commonly distributed risk factors in the studied individuals having rates of n=169(70%) and n=103(43%) respectively. Similarly contact with jaundice person (CJP) also found different in both group of participants. Distributions for other risk factors such as dentist visits, surgery, hospitalization etc. are shown in (Figure no. 4.13). Chi square distribution showed probability value of 0.001 and hence statistically significant.

4.5.10.2. Distribution of risk factors among male and female participants

Gender based differentiation of risk factors among participants revealed that most of the studied risk factors were equally distributed, however skin piercing and beauty parlor visit in females, whereas barber visits in males were the most frequent risk factors in both gender. Rates for skin piercing in female and barber visits in male were n=87(100%), n=210(100%) respectively. Distributions for the rest of the risk factors are shown in (Figure no. 4.14). Chi square distribution showed probability value (p) of <0.021, and hence the association was found to be statistically significant.

Figure 4.13. Observed risk factors among studied participants

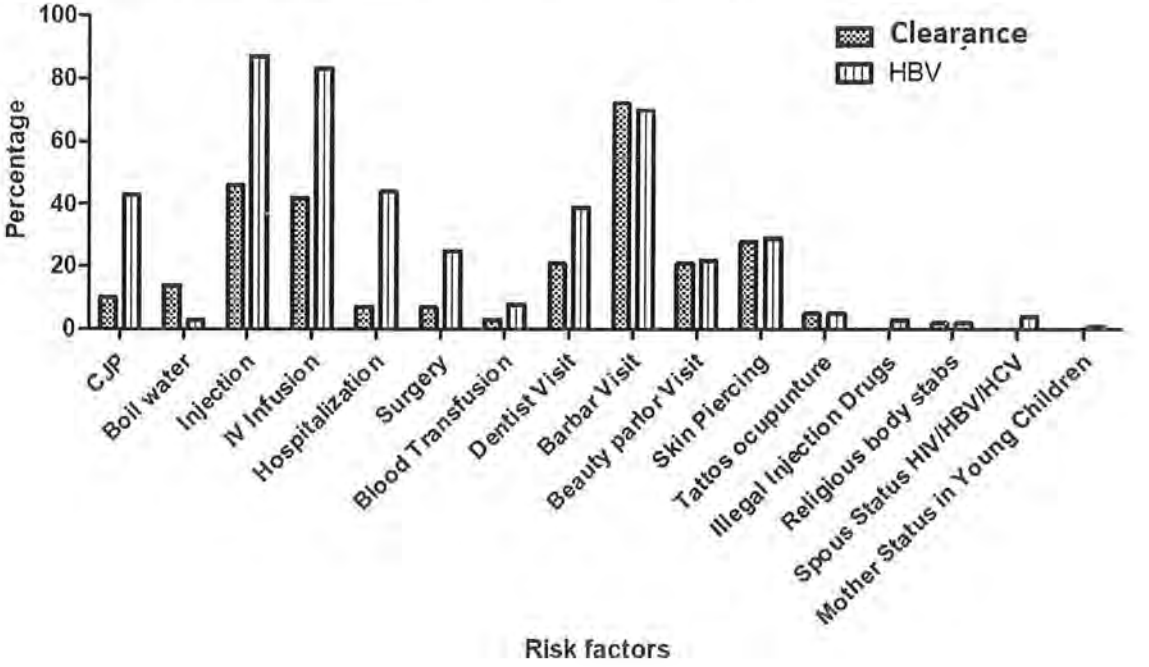
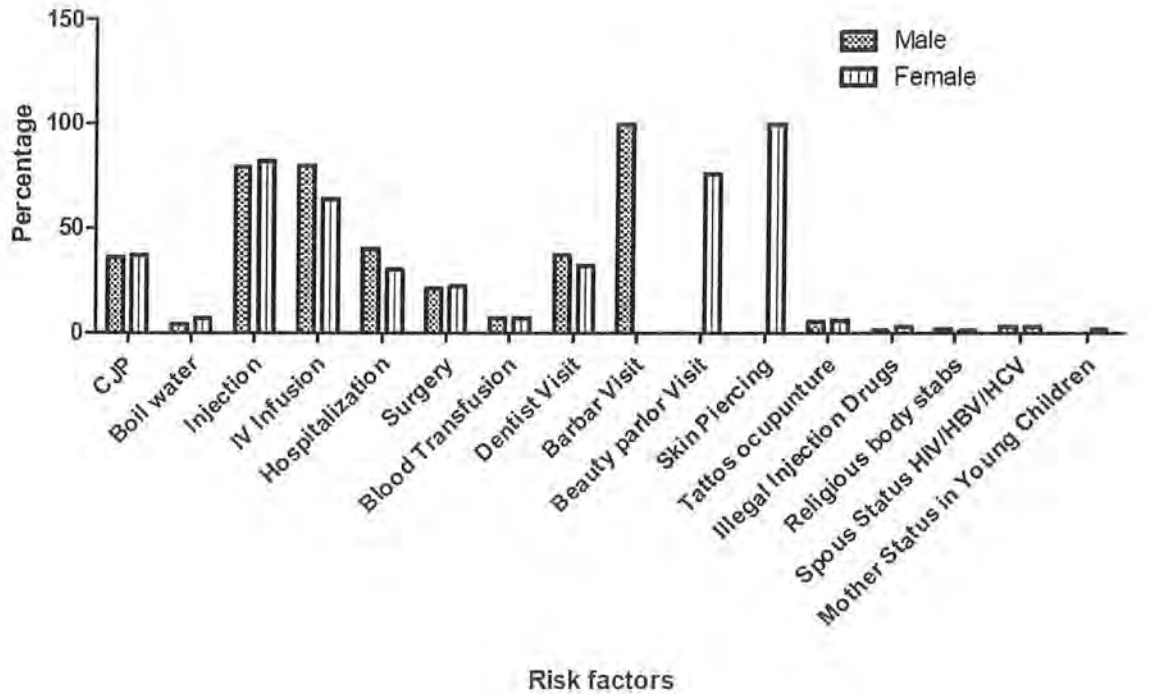


Figure 4.14. Distribution of risk factors among male and female participants



4.6.1. Characteristics of clearance group and Hepatitis B cohort

The demographic, biochemical and clinical attributes of HBV infected patients included in this study and clearance control are presented in **Table 4.6**. The median age of the patients was 29 years and 70.4% were male. Patients who have chronic HBV infection had significantly higher alanine transaminase (ALT) (P<0.001) and aspartate aminotransferase (AST) (P=0.036) but not total bilirubin (P=0.970) as compared to clearance control.

Table 4.6. Baseline characteristics of clearance group and hepatitis B cohorts

Variables	Clearance (n=57)	Hepatitis B (n=240)	*p* value
Age (years)[a]	28 (12 - 67)	29 (4 - 82)	0.405
Gender (male/female)[a]	41/16 (71.9%)	169/71 (70.4%)	0.873
ALT(IU/L)[a]	22 (11 - 42)	59(11 - 680)	**0.0001***
AST(IU/L)[a]	31 (22 - 42)	56 (15 - 459)	**0.036***
Total bilirubin (mg/dL)	0.7 (0.5 – 1.5)	0.8 (0.3 – 6.1)	0.970
Cholesterol (mg/dL)	135(80 - 229)	141 (78 - 280)	0.684
TG (mg/dL)	164 (98 - 307)	176 (92 - 351)	0.099
Platelets (x 10^9 /L)	275 (180 - 410)	231 (95 - 415)	**0.0001***

4.6.2. Characteristics of male and female participants

A total of 210(71%) Males and 87(29%) females were recruited in this study. Various characteristic of the both gender are presented in **Table 4.7.** No statistically significant difference of biochemical, demographic and clinical variable found among male and female patients. Male patients have mean age 28 years was lower as compared to female patients with mean age of 31 years.

Table 4.7. Gender base characteristics of studied participants

Variables	Male (n=210)	Female (n=87)	*p* value
Age (years)[a]	28 (4 - 82)	31 (6 - 71)	**0.050***
ALT(IU/L)[a]	48(11 - 355)	63 (11 - 680)	0.378
AST(IU/L)[a]	50 (15 - 280)	55 (18 - 459)	0.625
Total bilirubin (mg/dL)	0.7 (0.3 – 4.0)	0.9 (0.4 – 6.0)	0.511
Cholesterol(mg/dL)	140 (78 - 280)	141 (80 - 240)	0.529
TG (mg/dL)	174 (92 - 351)	173 (95 - 312)	0.595
Platelets (x 10^9 /L)	237 (95 - 415)	246 (128 - 400)	0.304

4.7.1. *STAT4* rs7574865 but not *IFNL3* is associated with risk of HBV chronicity

The genotype distribution of *STAT4* rs7574865 and *IFNL3* rs12979860 in HBV infected patients and in the clearance group of Pakistani population is shown in **Table 4.8.** Genotype distribution was in Hardy-Weinberg equilibrium in both groups. We assessed the association of rs7574865 with HBV chronicity. The minor allele (T) frequency (MAF) of rs7574865 was 0.22, significantly lower than that observed in our clearance cohort (MAF 0.33 (p=0.04) and also using the recessive model (p=0.02). In contrast, the genotype distribution of *IFNL3* rs12979860 was not significantly different between HBV-infected patients and control group (0.25 vs. 0.21, p=0.3) and also using the dominant model (p=0.2).

Table 4.8. rs7574865 and rs12979860 genotype distribution in the Clearance and HBV cohorts

Genotype	Clearance(n=57)		Hepatitis B(n=240)		*p* value
	Frequency	Percentage	Frequency	Percentage	
STAT4 rs7574865					
GG	25	43.9%	145	60.4%	**0.048**
GT	26	45.6%	83	34.6%	
TT	6	10.5%	12	5.0%	
GG	25	43.9%	145	60.4%	**0.026**
GT/TT	32	56.1%	95	39.6%	
IFNL3 rs12979860					
CC	36	63.2%	132	55%	0.342
CT	17	29.8%	96	40%	
TT	4	7.0%	12	5.0%	
CC	36	63.2%	132	55.0%	0.300
CT/TT	21	36.8%	108	45.0%	

4.7.2. *STAT4* rs7574865 and IFNL3 rs12979860 genotype association with gender

The genotype distribution of *STAT4* rs7574865 and *IFNL3* rs12979860 in male and female patients in Pakistani population is shown in **Table 4.9**. Genotype distribution was in Hardy-Weinberg equilibrium in both groups. We also analyzed the association *STAT4* rs7574865 with male and female group. The minor allele (T) frequency (MAF) of rs7574865 was nearly equal in both male and female groups (MAF 0.22 vs. 0.22 p=0.41) and also using the recessive model (p=0.66). Similarly, the genotype distribution of *IFNL3* rs12979860 was not significantly different between male and female patients (0.25 vs. 0.23, p=0.61) and also using the dominant model (p=0.47).

Table 4.9. Gender base distribution of *STAT4* rs7574865 and *IFNL3* rs12979860 genotype

Genotype	MALE (n=169)		FEMALE(n=71)		*p* value
	Frequency	Percentage	Frequency	Percentage	
STAT4 rs7574865					
GG	104	61.5%	41	57.7%	0.413
GT	55	32.5%	28	39.4%	
TT	10	5.9%	2	2.8%	
GG	104	61.5%	41	57.7%	0.665
GT/TT	65	38.5%	30	42.3%	
IFNL3 rs12979860					
CC	90	53.3%	42	59.2%	0.614
CT	71	42.0%	25	35.2%	
TT	8	4.7%	4	5.6%	
CC	90	53.3%	42	59.2%	0.477
CT/TT	79	46.7%	29	40.8%	

4.8. rs7574865 and rs12979860 regression analysis in clearance group and chronic HBV patients

The association of rs7574865 genotype among clearance and HBV cohort remained significant in multiple logistic regression analysis after adjusting for age and gender (Odds ratio (OR) = 2.0, 95% CI: 1.11–3.61, p= 0.02). In contrast, the association of rs12979860 was not significantly different between both group in multiple logistic regression analysis after adjusting for the same variables above (OR: 0.7, 95% CI: 0.39-1.29, p=0.2) **Table 4.10**.

Table 4.10. Association of rs7574865 and rs12979860 genotype in HBV persistence

Genotype	OR (95%CI)	*p* value
STAT4 rs7574865		
GG	1	
GT	1.8 (0.98 - 3.35)	**0.056**
TT	2.9 (0.99 - 8.43)	**0.051**
Adjusted	2.0 (1.11 - 3.61)	**0.021**
Dominant	0.5 (0.28 – 0.91)	**0.025**
GG	1	
GT/TT	1.9 (1.09 - 3.50)	**0.025**
IFNL3 rs12979860		
CC	1	
CT	0.6 (0.34 - 1.22)	0.182
TT	1.2 (0.37 - 4.01)	0.741
Adjusted	0.7 (0.39 – 1.29)	0.271
Dominant	1.4 (0.77 – 2.54)	0.265
CC	1	
CT/TT	0.7 (0.39 - 1.29)	0.265

4.9.1. Association of *STAT4* rs7574865 genotype with clinical variables

We further explored, if baseline clinical variables differed between HBV infected patients respective their *STAT4* rs7574865 genotype; the results are presented in **Table 4.11**. While there was no evidence of significant association between rs7574865 genotype, comparing minor genotype to other genotypes (GG versus GT versus TT) and any other clinical variables (i.e. age, ALT, AST gender, HBV-DNA cholesterol, TG, Platelets and total bilirubin).

4.9.2. Association of *IFNL3* rs12979860 genotype with clinical variables

We scrutinized whether baseline clinical variables differed between HBV infected patients respective their *IFNL3* rs12979860 genotype; the results are presented in **Table 4.12**. We detected that subjects with *IFNL3* rs12979860 CT genotype have significantly low levels of cholesterol when compared to those with CC and TT genotype, while here was no evidence of significant association between rs12979860 genotype (CC versus CT versus TT) with any of the other clinical variables mentioned above.

Table 4.11. Association of clinical variables with *STAT4* rs7574865 genotype

STAT4 rs7574865	GG	GT	TT	*p* value
Age	28.0±13.3	30.5±13.7	30.1±15.1	0.337
HBV-DNA log IU	5.2±2.2	5.4±2.2	4.9±1.8	0.797
ALT (IU/L)	45.6±45.2	61.9±101.4	55.0±70.2	0.185
AST (IU/L)	47.4±38.4	55.9±63.3	61.1±61.3	0.278
T.bilirubin (mg/dl)	0.77±0.35	0.88±0.70	0.69±0.34	0.155
Cholesterol (mg/dL)	141.7±45.5	139.1±43.6	134.8±40.6	0.771
TG (mg/dL)	176.9±52.2	169.9±45.5	168.0±51.9	0.459
Platelets (x 10^9 /L)	239.6±60.7	239.7±62.2	238.1±65.8	0.995

Table 4.12. Association of clinical variables with *IFNL3* rs12979860 genotype

IFNL3 rs12979860	CC	CT	TT	*p* value
Age	28.8±13.3	29.0±13.7	32.1±15.5	0.644
HBV-DNA log IU	5.6±2.3	4.9±2.1	4.4±0.8	0.140
ALT (IU/L)	50.7±70.5	52.8±74.8	62.6±81.7	0.818
AST (IU/L)	50.4±47.8	51.6±52.8	60.5±62.0	0.744
Total bilirubin (mg/dl)	0.76±0.37	0.88±0.68	0.76±0.29	0.135
Cholesterol (mg/dL)	146.1±47.3	130.8±36.1	148.2±56.2	**0.014**
TG (mg/dL)	178.0±53.5	167.4±41.1	174.7±62.6	0.214
Platelets (x 10^9 /L)	234.2±60.5	248.1±63.1	235.8±55.1	0.172

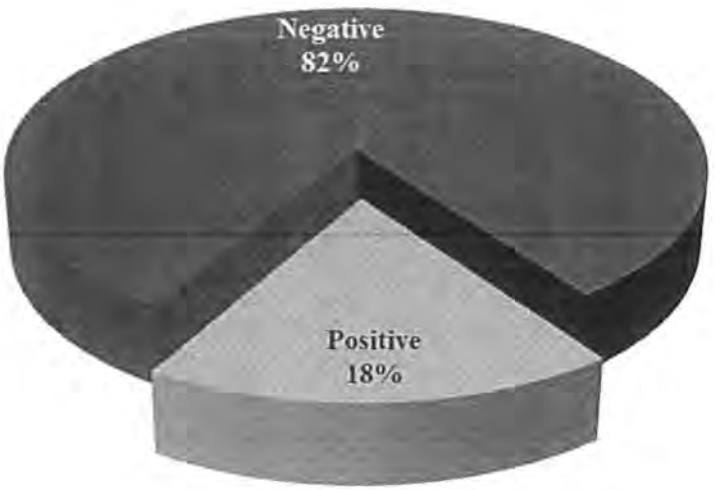
B2. Group of patients with steatosis versus those without steatosis.

This part of study includes patients with steatosis and non-steatosis and their relative characteristics. Healthy control individuals (57) were also enrolled. Five genetic variants were analysed among this two categories of patients. Three SNP were considered specifically for patients with steatosis including TM6SF2, PNPLA3 and MBOAT7. The results of each variant are below.

4.10.1. Prevalence of steatosis and non-steatosis HBV patients

Ultrasonography and APRI test were used to evaluate the steatosis and non-steatosis patients. Among the patients with chronic HBV, 44 (18%) were found to have steatosis and n=196 (82%) did not have steatosis (Figure 4.15).

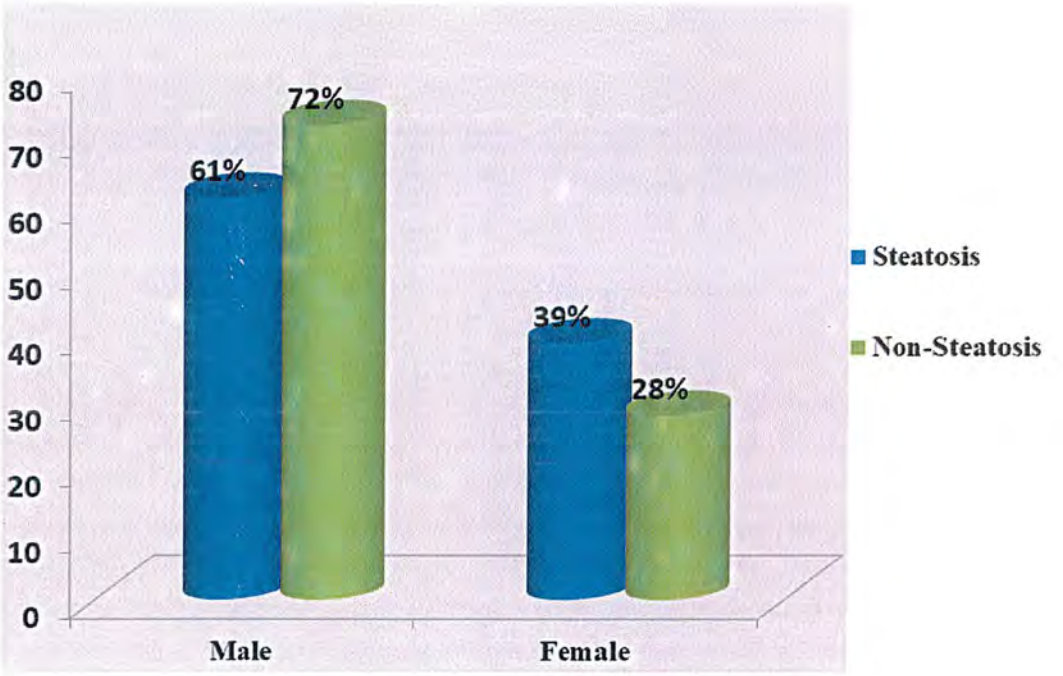
Figure 4.15 Chart representing Non-steatosis and steatosis group of chronic HBV patients



4.10.2. Gender base prevalence of Steatosis and Non-Steatosis HBV patients

Further we analyzed gender based prevalence of Steatosis and Non-Steatosis patients. The steatosis group comprised of n=27 (61%) male patients, while n=17 (39%) of the patients were female respectively. Similarly, the Non-Steatosis infected group was comprised of n=142 (72%) of males and n=54 (28%) of female patients (Figure 4.16). (p=0.149, statistically not significant)

Figure 4.16.Gender base distribution of Steatosis and Non-Steatosis patients

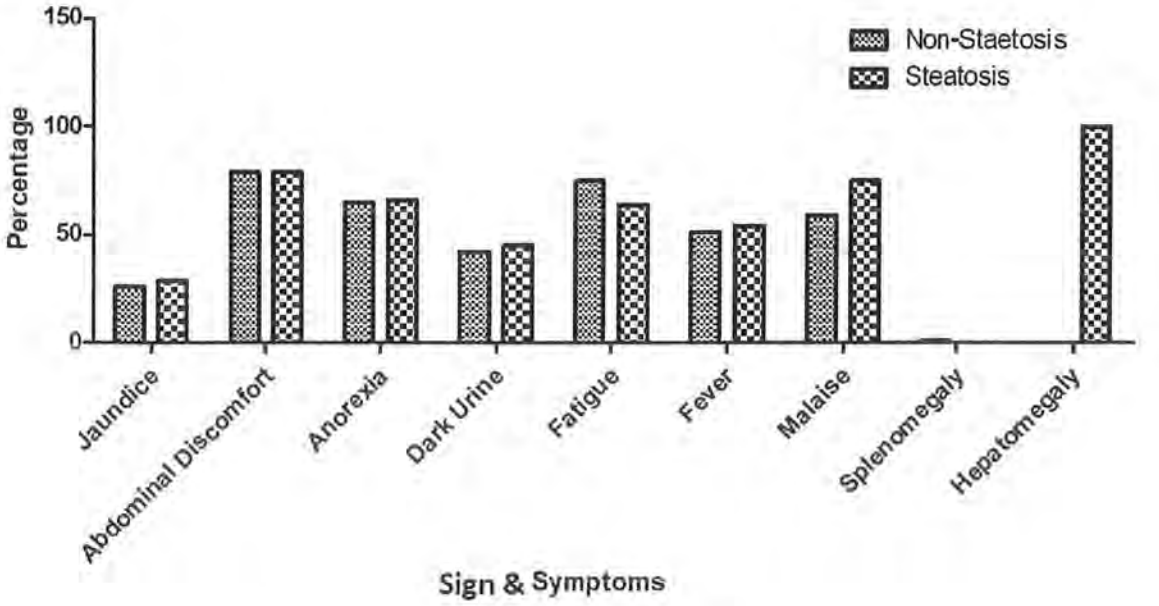


4.10.3. Distribution of signs and symptoms in Non-steatosis and steatosis patients

Signs and symptoms differentiation in Non-steatosis and steatosis participants revealed that hepatomegaly was the most prevalent sign in steatosis as compared to Non-steatosis, n=44(100%) vs n=0(0%). Some of the major symptom slightly varied among Non-

steatosis and steatosis were malaise and fatigue had the prevalence rates among steatosis were n=33(75%) and n=28(64%) as compared to Non-steatosis n=115(59%) and n=147(75%) respectively. Other studied symptoms like abdominal discomfort, anorexia were almost equally distributed among Non-steatosis and steatosis individuals as shown in (Figure no. 4.17). Chi square distribution showed probability value of 0.272 and hence statistically not significant.

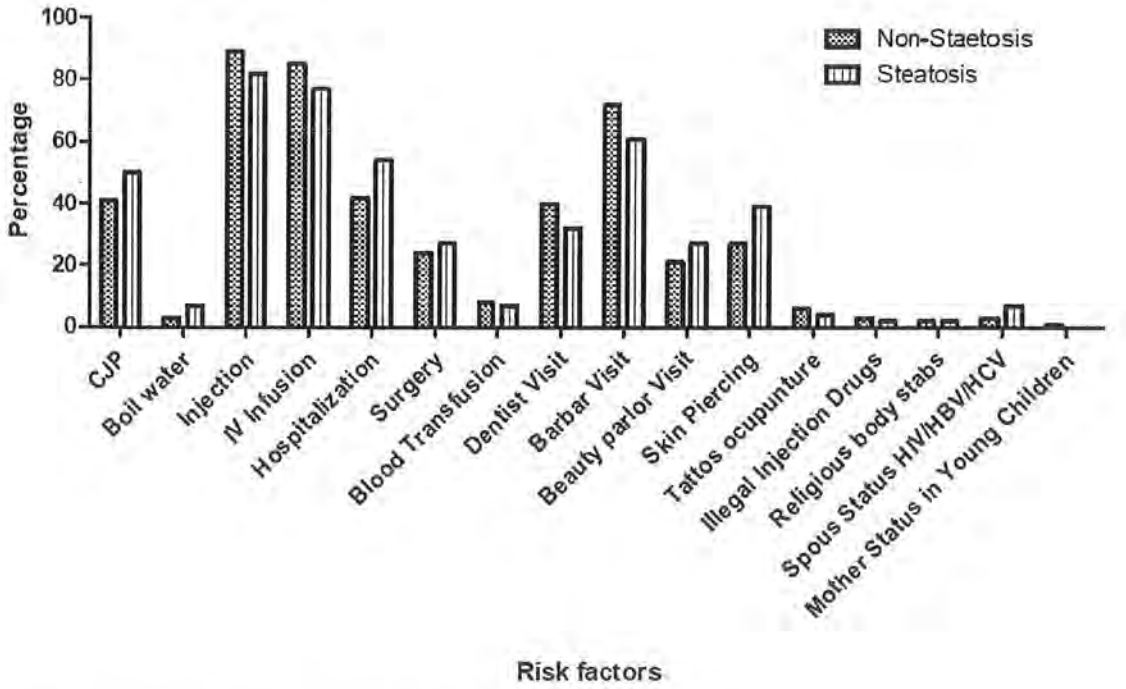
Figure 4.17. Signs and Symptoms distribution among No-steatosis and steatosis patients



4.10.4. Distribution of risk factors among Non-steatosis and steatosis group

Next we analysed risk factors among steatosis and Non-steatosis participants and the data revealed that most of the studied risk factors were equally distributed, however some risk factors were slightly different between two group, like skin piercing and hospitalization in steatosis, were high n=17(39%), and n=24(54%), as compare to Non-steatosis group n=54(27%) and n=82(42%) respectively. Other risk factors were equally distributed among both groups. Distributions for the rest of the risk factors are shown in (Figure no. 4.18). Chi square distribution showed probability value (p) of <0.452, and hence the association was found to be statistically not significant.

Figure 4.18. Risk factors analysis among Non-steatosis and steatosis group



4.11. Characteristics of Non-Steatosis and Steatosis cohort

The clinical attributes of Non-Steatosis and Steatosis cohort included in this study are presented in **Table 4.13**. Individuals presenting with steatosis had significantly higher serum cholesterol ($P<0.001$) and triglyceride ($P=0.014$) concentrations as compared to individuals with no steatosis. Similarly, ALT and AST level also varied between Non-steatosis and patients with steatosis.

Table 4.13. Baseline characteristics of Non-Steatosis and Steatosis cohorts

Variables	Non-Steatosis (n=196)	Steatosis (n=44)	*p* value
Age (years)[a]	26 (4 - 75)	44 (17 - 82)	**0.0001***
Gender (male/female)[a]	142/54 (72.4%)	27/17 (61.4%)	0.149
ALT(IU/L)[a]	33(11 - 211)	175 (25 - 680)	**0.0001***
AST(IU/L)[a]	35 (15 - 103)	149 (70 - 459)	**0.0001***
Total bilirubin (mg/dL)	0.7 (0.3 – 3.8)	0.9 (0.4 – 6.1)	0.140
Cholesterol(mg/dL)	131 (78 - 260)	138(78 - 280)	**0.001***
TG (mg/dL)	173 (92 - 351)	189(120 - 312)	**0.014***
Platelets (x 10^9 /L)	245 (150 - 415)	169 (95-243)	**0.0001***

4.12.1. *PNPLA3* rs738409 and *TM6SF2* but not *MBOAT* rs641738 is associated with hepatic steatosis

Next, we explored the association of *PNPLA3*, *TM6SF2* and *MBOAT7* with hepatic steatosis. The genotype distribution of *PNPLA3* rs738409, *TM6SF2* and *MBOAT7* in chronic HBV infected patients and in hepatic steatosis Pakistani population is shown in **Table 4.14**. The rs738409 GG genotype, observed in 3% of patients, was associated with steatosis (0.27 vs. 0.21, p=0.033). The rs58542926 TT genotype, observed in 4.6% of patients, the minor allele (T) frequency (MAF) of *TM6SF2* rs58542926 was significantly higher in patients with steatosis compared to those without steatosis (0.11 vs. 0.05, p=0.024). In contrast, *MBOAT* rs641738 CC genotype, observed 25.8% of patients, the genotype distribution of *MBOAT* rs641738 was not significantly different between chronic HBV infected patients and in steatosis cohort (0.49 vs. 0.47, p=0.666) and also using the recessive model (p=0.448).

Table 4.14. Genotype variants distribution in Steatosis and Non- steatosis cohorts

Genotype	Non-Steatosis (n=196)		Steatosis (n=44)		*p* value
	Frequency	Percentage	Frequency	Percentage	
PNPLA3 rs738409					
CC	94	48.0%	28	63.6%	0.033*
CG	97	49.4%	13	29.5%	
GG	5	2.6%	3	6.9%	
CC	94	48.0%	28	63.6%	0.068
CG/GG	102	52.0%	16	36.4%	
TM6SF2 rs58542926					
CC	181	92.3%	39	88.6%	0.024*
CT	9	4.6%	0	0.0%	
TT	6	3.1%	5	11.4%	
CC	181	92.3%	39	88.6%	0.380
CT/TT	15	7.7%	5	11.4%	
MBOAT7 rs641738					
CC	53	27.0%	9	20.5%	0.666
CT	94	48.0%	23	52.3%	
TT	49	25.0%	12	27.2%	
CC	53	27.0%	9	20.5%	0.448
CT/TT	143	73.0%	35	79.5%	

4.12.2. *STAT4* rs7574865 and IFNL3 rs12979860 genotype association with steatosis

The genotype distribution of *STAT4* rs7574865 and *IFNL3* rs12979860 in Non-steatosis and steatosis patients in Pakistani population is shown in **Table 4.15**.Genotype distribution was in Hardy-Weinberg equilibrium in both groups. Next we assessed the association of rs7574865 with steatosis. The minor allele (T) frequency (MAF) of rs7574865 was 0.30, higher than that observed in our Non-steatosis cohort (MAF 0.20 (p=0.11) and also using the recessive model (p=0.06). Similarly, the genotype

distribution of *IFNL3* rs12979860 was not significantly different between Non-steatosis and steatosis patients (0.25 vs. 0.23, p=0.60) and also using the dominant model (p=0.61).

Table 4.15. rs7574865 and rs12979860 genotype distribution in Non-steatosis and steatosis patients

Genotype	Non-Steatosis (n=196)		Steatosis (n=44)		*p* value
	Frequency	**Percentage**	**Frequency**	**Percentage**	
STAT4 rs7574865					
GG	124	63.3%	21	47.7%	**0.111**
GT	64	32.7%	19	43.2%	
TT	8	4.1%	4	9.1%	
GG	124	63.3%	21	47.7%	0.062
GT/TT	72	36.7%	23	52.3%	
IFNL3 rs12979860					
CC	106	54.1%	26	59.1%	0.609
CT	81	41.3%	15	34.1%	
TT	9	4.6%	3	6.8%	
CC	106	54.1%	26	59.1%	0.616
CT/TT	90	45.9%	18	40.9%	

4.12.3. *PNPLA3* rs738409, *TM6SF2* and *MBOAT* rs641738 genotype association with gender

The genotype distribution of *PNPLA3* rs738409 *TM6SF2* rs58542926 and *MBOAT* rs641738 in male and female patients in Pakistani population is shown in **Table 4.16**. Genotype distribution was in Hardy-Weinberg equilibrium in both male and female groups. The rs738409 GG genotype, observed in 3% of patients, was not significantly different between both gender (0.28 vs. 0.22, p=0.148). The minor allele (T)

frequency (MAF) of *TM6SF2* rs58542926 was not significantly varied in male and female patients (0.07 vs. 0.04, p=0.606). Similarly the distribution of *MBOAT* rs641738 was not significantly different between male and female individuals (0.47 vs. 0.54, p=0.369) and also using the recessive model (p=0.197).

4.13. *PNPLA3* rs738409 and *TM6SF2* but not *MBOAT* rs641738is associated with risk of HBV chronicity

The genotype distribution of *PNPLA3* rs738409, *TM6SF2* and*MBOAT7* in HBV infected patients and in the healthy Pakistani population is shown in **Table 4.17**.Genotype distribution was in Hardy-Weinberg equilibrium in both groups. The minor allele (G) frequency (MAF) of *PNPLA3* rs738409 was 0.26, significantly higher than that observed in our healthy cohort (MAF 0.16, p=0.031), and also using the recessive model (p=0.011). The minor allele (T) frequency (MAF) of *TM6SF2* rs58542926 was 0.06, significantly higher than that observed in our healthy cohort (MAF 0.00 (p=0.078), and also using the dominant model (p=0.018). In contrast, the genotype distribution of *MBOAT* rs641738 was not significantly different between HBV-infected patients and healthy control (0.49 vs. 0.41, p=0.137) and also using the recessive model (p=0.500).

Table 4.16. Genotype variants distribution in male and female patients.

Genotype	MALE (n=169)		FEMALE (n=71)		*p* value
	Frequency	Percentage	Frequency	Percentage	
PNPLA3 rs738409					
CC	79	46.7%	43	60.6%	0.148
CG	84	49.7%	26	36.6%	
GG	6	3.6%	3	2.8%	
CC	79	46.7%	43	60.6%	0.066
CG/GG	90	53.3%	28	39.4%	
TM6SF2 rs58542926					
CC	153	90.5%	67	94.4%	0.606
CT	7	4.1%	2	2.8%	
TT	9	5.3%	2	2.8%	
CC	153	90.5%	67	94.4%	0.445
CT/TT	16	9.5%	4	5.6%	
MBOAT7 rs641738					
CC	48	28.4%	14	19.7%	0.369
CT	80	47.3%	37	52.1%	
TT	41	24.3%	20	28.2%	
CC	48	28.4%	14	19.7%	0.197
CT/TT	121	71.6%	57	80.3%	

Table 4.17. Genotype variants distribution in the healthy and hepatitis B cohorts

Genotype	Healthy (n=57)		Hepatitis B (n=240)		*p* value
	Frequency	Percentage	Frequency	Percentage	
PNPLA3 rs738409					
CC	40	70.2%	122	50.8%	0.031*
CG	16	28.1%	110	45.8%	
GG	1	1.8%	8	3.3%	
CC	40	70.2%	122	50.8%	0.011*
CG/GG	17	29.8%	118	49.2%	
TM6SF2 rs58542926					
CC	57	100.0%	220	91.7%	0.078
CT	0	0.0%	9	3.8%	
TT	0	0.0%	11	4.6%	
CC	57	100.0%	220	91.7%	0.018*
CT/TT	0	0.0%	20	8.3%	
MBOAT7 rs641738					
CC	12	21.1%	62	25.8%	0.137
CT	23	40.4%	117	48.8%	
TT	22	38.6%	61	25.4%	
CC	12	21.1%	62	25.8%	0.500
CT/TT	45	78.9%	178	74.2%	

4.14.1. *PNPLA3, TM6SF2* and *MBOAT7* regression analysis in Steatosis and Non-Steatosis patients

Age, serum cholesterol and triglycerides tend to be higher in patients with hepatic steatosis, though it was significant, while significant association of other clinical variable like ALT, AST and platelets were also noticed between patients with and without hepatic steatosis **Table 4.13.** Although the association of rs738409, genotype among Non-steatosis and steatosis patients remained insignificant in multiple logistic regression

analysis (Odds ratio (OR) = 1.8, 95% CI: 0.96–3.73, p= 0.06). In addition, the association of rs641738 was not significantly different between both group in multiple logistic regression analysis (OR: 0.6, 95% CI: 0.31-1.54, p=0.36). Similarly the association of rs58542926 was not significantly different between steatosis and non-steatosis group in multiple logistic regression analysis (OR: 0.6, 95% CI: 0.22-1.88, p=0.42)**Table 4.18.**

Table 4.18. Association of rs738409, rs641738 and rs58542926 genotype in hepatic steatosis

	Non-Steatosis vs Steatosis	
Genotype	**OR (95%CI)[a]**	***p* value**
PNPLA3 rs738409		
CC	1	
CG	2.2 (1.08 – 4.55)	0.029*
GG	0.4 (0.11 – 2.20)	0.358
CC	1	1
CG/GG	1.8 (0.96 – 3.73)	0.063
MBOAT7 rs641738		
CC	1	
CT	0.6 (0.29 – 1.60)	0.395
TT	0.6 (0.26 – 1.78)	0.449
CC	1	
CT/TT	0.6 (0.31 – 1.54)	0.369
TM6SF2 rs58542926		
CC	1	
CT	0.6 (0.22 – 1.88)	0.585
TT	0.2 (0.07 – 0.89)	**0.032***
CC	1	
CT/TT	0.6 (0.22 – 1.88)	0.424

4.14.2. *STAT4* rs7574865 and IFNL3 rs12979860 regression analysis in Steatosis and Non-Steatosis patients

Although the association of rs7574865 genotype among Non-steatosis and steatosis patients remained insignificant in multiple logistic regression analysis (Odds ratio (OR) = 0.5, 95% CI: 0.27–1.02, p= 0.05). In addition, the association of rs12979860 was not significantly different between both group in multiple logistic regression analysis (OR: 1.2, 95% CI: 0.63-2.38, p=0.54) **Table 4.19**.

Table 4.19. Association of *STAT4* rs7574865 and *IFNL3* rs12979860 genotype in Steatosis

Genotype	OR (95%CI)	*p* value
STAT4 rs7574865		
GG	1	
GT	0.5 (0.28 – 1.13)	0.111
TT	0.3 (0.09 – 1.22)	0.099
GG	1	
GT/TT	0.5 (0.27 – 1.02)	0.059
IFNL3 rs12979860		
CC	1	
CT	1.3 (0.65 – 2.66)	0.430
TT	0.7 (0.18 – 2.91)	0.662
CC	1	
CT/TT	1.2 (0.63 – 2.38)	0.547

4.15. *PNPLA3,* rs738409 and *MBOAT* rs641738 regression analysis in healthy and chronic HBV patients

Further we analysed *PNPLA3* rs738409, and *MBOAT* rs641738 by regression analysis and rs738409 genotype association remained significant in multiple logistic regression analysis after adjusting for age and gender (OR= 0.41, 95% CI: 0.22 – 0.78, p= 0.006) **Table 4.20**. In contrast, the association of *MBOAT* rs641738 was not significantly

different between both group in multiple logistic regression analysis after adjusting for the same variables (OR: 1.3, 95% CI: 0.66 – 2.70, p=0.417) **Table 4.20**.

Table 4.20. Association of rs738409 and rs641738 genotype in HBV persistence

	Healthy vs Chronic Hepatitis B	
Genotype	**OR (95%CI)[a]**	***p* value**
PNPLA3 rs738409		
CC	1	
CG	0.4 (0.23 - 0.83)	0.012*
GG	0.3 (0.04 - 3.14)	0.370
Adjusted	0.4 (0.22 –0.78)	0.006*
Dominant	2.2 (1.22 – 4.23)	0.009*
CC	1	1
CG/GG	0.4 (0.23 – 0.81)	0.009*
MBOAT7 rs641738		
CC	1	
CT	1.0 (0.47 – 2.17)	0.968
TT	1.8 (0.84 – 4.09)	0.121
Adjusted	1.3 (0.66 – 2.70)	0.417
Dominant	0.7 (0.38 – 1.54)	0.454
CC	1	
CT/TT	1.3 (0.64 – 2.62)	0.454

4.16. Association of *PNPLA3, TM6SF2* and *MBOAT7* genotype with clinical variables

Next, we explored if baseline clinical variables differed between HBV infected patients according to *PNPLA3* rs738409 genotype; the results are presented in **Table 4.21**. We observed that subjects with rs738409 CG genotype have significantly lower ages levels compared to those with CC and GG genotype (p=0.009), we also observed that the

subject with rs738409 TT genotype have higher ALT level as compared to CC and CT genotype (p=0.027).Next we also found that subject with rs641738 CC genotype have significantly higher cholesterol level as compared to CT and TT genotype (p=0.014). While here was no evidence of significant association between *PNPLA3* rs738409, *TM6SF2* rs58542926 or *MBOAT7* rs641738 genotype with any of the other clinical variables i.e., gender, AST, HBV-DNA, total bilirubin, Platelets and TG **Table 4.21.1.** and **Table 4.21.2.**

Table 4.21.1. Association of clinical variables with various alleles.

PNPLA3 rs738409	**CC**	**CG**	**GG**	***p* value**
Age	31.1±14.4	26.3±12.1	31.8±10.8	**0.009***
HBV-DNA Log IU	5.1±2.1	5.5±2.3	5.1±2.1	0.544
ALT (IU/L)	51.9±60.1	50.6±85.7	80.4±81.0	0.492
AST (IU/L)	53.0±48.4	49.0±52.0	69.2±64.3	0.400
Total bilirubin (mg/dL)	0.79±0.41	0.83±0.63	0.68±0.19	0.616
Cholesterol (mg/dL)	144.6±45.9	135.4±42.6	133.8±38.3	0.199
TG (mg/dL)	177.5±50.6	167.3±46.0	198.6±73.6	0.072
Platelets (x 10^9 /L)	240.6±60.3	2389.2±63.3	225.4±58.6	0.769

Table 4.21.2. Association of clinical variables with various alleles

TM6SF2 rs58542926	CC	CT	TT	*p* value
Age	29.2±13.6	21.2±8.3	31.4±15.9	0.185
HBV-DNA Log IU	5.3±2.2	5.1±2.4	4.6±1.8	0.680
ALT (IU/L)	50.2±71.1	41.8±32.6	79.0±88.5	**0.027***
AST (IU/L)	50.5±49.7	39.8±17.9	83.5±75.0	0.081877
Total bilirubin (mg/dL)	0.80±0.51	0.89±0.58	0.81±0.31	0.881
Cholesterol (mg/dL)	140.5±44.2	116.7±36.4	157.6±50.3	0.122
TG (mg/dL)	174.7±50.0	137.5±29.2	188.0±47.1	**0.057**
Platelets (x 10^9 /L)	240.0±60.9	237.8±26.6	230.3±91.6	0.875
MBOAT7rs641738	**CC**	**CT**	**TT**	***p* value**
Age	26.8±13.4	30.6±13.4	28.4±13.7	0.132
HBV-DNA log IU	5.4±2.2	5.2±2.2	5.1±2.3	0.784
ALT (IU/L)	44.9±48.4	56.4±90.9	51.5±53.2	0.545
AST (IU/L)	43.8±33.9	54.7±60.0	52.5±44.5	0.318
Total bilirubin (mg/dl)	0.82±0.50	0.80±0.57	0.80±0.39	0.935
Cholesterol (mg/dL)	149.4±44.8	142.2±45.1	129.2±40.9	0.014
TG (mg/dL)	180.2±44.0	177.7±54.2	161.4±45.0	**0.027***
Platelets (x 10^9 /L)	233.1±51.9	243.1±64.6	239.3±63.8	0.531

4.17. Association of clinical variables with HBV-DNA levels

Finally, we looked if any of the studied clinical variables, namely age, gender, ALT, AST, total bilirubin cholesterol, TG or platelets was correlated with HBV-DNA levels. Patients with high HBV DNA level have significantly high cholesterol (p=0.044) and triglyceride (p=0.010) as compared to low HBV DNA level. While none of the other variables was associated with HBV-DNA levels **Table 4.22**.

Table 4.22. Clinical variables association with HBV DNA load.

Parameter	Patients with low HBV DNA Levels (<1000MU/mL)	Patients with high HBV DNA Levels (>1000MU/mL)	*p* value
Age	29.51	29.25	0.889
ALT (IU/L)	60.81	58.36	0.819
AST (IU/L)	55.12	56.76	0.825
T.bilirubin (mg/dl)	0.90	0.78	0.124
Cholesterol (mg/dL)	133.63	146.16	**0.044***
TG (mg/dL)	164.94	182.34	**0.010***
Platelets (x 10^9 /L)	232.77	230.36	0.763

Discussion

Viral hepatitis remains one of the most formidable challenges in Pakistan. National vaccination program against hepatitis B, as a part of global effort to reduce the burden of the disease has already been launched in the country. However, lack of national surveillance program and large scale community based studies may undermine the outcome of immunization program in the long run, particularly because there is a scarcity of the data about prevalence of HBV/HDV infections among general population of Pakistan across different regions. With a particular focus on outpatient department patients with no prior history of any kind of hepatitis altogether 946 patients were screened for HBV infection. Out of these 22.45% were found positive for HBsAg. Previously, different prevalence rates of HBV infection were reported from Pakistan ranging from minimal 5% to maximal 41.26% %(Khan *et al.* 2012; Ali *et al.* 2014)

In this study, serological scrutiny confirmed that 86.79% of the HBV infected patients were positive for anti-HBc-total. Detection of both HBsAg and anti-HBc-total has been documented in chronic patients while HBeAg may or may not be detected in such patients (Horyat *et al.*2006). Hence, detection of anti-HBc-total, alone may not suffice an evidence for chronic HBV infection in these patients. However, detection of anti-HBc-total in the presence of viral DNA in such patients may reflect active presence of HBV infection that is likely to proceed towards chronic stage. In this study, viral DNA was detected in 53.77% of the anti-HBc-total positive patients which are at the higher risk of developing chronic disease. Moreover, due to the active state of their infection these individual pose a risk for further dissemination of HBV infection. While majority of the HBsAg positive patients were also positive for anti-HBc-total, 25% showed presence of anti-HBc-IgM, which is considered as an important serological marker to diagnose recent HBV infections, in so-called "window period".

Among HBsAg positive samples, screening for co-infection of HDV was one the most important aspect of this study. Seroprevalence of HDV varies greatly across different countries, for example, in Turkey, Saudi Arabia and Bangladesh prevalence rates were observed 5.2%, 3.3% and 24.4%, respectively (Din *et al.*2014; Alayian *et al.*2005).

Previously, only few studies have been conducted to assess the seroprevalence of HDV in Pakistan and demonstrated some inconsistencies (Mumtaz *et al.* 2005; Shaikh *et al.*2011). Observed differences in seroprevalence of HDV in these studies were attributed to several factors, including different geographical locations, population differences and detection methods used (RNA or anti-HDV). Based on anti-HDV detection prevalence rate for HDV infection in this study appeared to be 10.84%. Notably, it is the first report from Pakistan which highlights the prevalence of HBV/HDV co-infection in a population with no prior history of viral hepatitis.

Prevalence of co-infection with HCV varies among infected HBV patients ranging from 10% to 20% (Kyitko *et al.*2013). HCV infections are reported among different groups of patients having organ transplantation, injection drug users and patients with beta-thalassemia (Reker *et al.* 2014; Pereira *et al.* 2013). Co-infection with HDV/HCV leads to deleterious clinical outcomes including cirrhosis and hepatocellular carcinoma (Kershenobich *et al.* 2011; CDC 2011. In our study 7.4% of HBsAg positive patients were co-infected with HCV.

Generally, a wide range of clinical manifestations are associated with HBV infections including asymptomatic sero-conversion, non-specific symptoms (anorexia, nausea, etc.) and extra hepatic symptoms. We observed significantly higher association of HBV infection with various symptoms, including abdominal discomfort, fatigue, anorexia, malaise, fever, dark urine, jaundice and splenomegaly. Abdominal discomfort was one of the most prominent symptoms associated with HBV infection in this study. Similarly, significant association of different risk factors and HBV infection was also observed. Overall use of injection was the major risk factor that followed intravenous infusion.

Globally around two billion individuals are exposed to HBV, which are consisted of 400 million chronic infections and 800, 000 deaths yearly. HBV is a non-cytopathic virus having a dsDNA genome and one of the foremost members of Hepadnaviridae family. HBV related complications can mostly be attributed to the host immunogenetic response against the virus (Sharkawy *et al.* 2018). As we know that HBV infection can result clinical manifestations like chronic liver diseases and liver carcinoma. The risks of

developing these clinical outcomes are mostly dependent upon various factors like, route of infection, HBV serotype and immune status of the host. The predominant transmission route for HBV infection is perinatal or horizontal route in Asian and African countries, however in European countries the HBV transmission is mainly attributed to sexual activities and injecting drugs use (Jefferies *et al.* 2018).

Pakistan is highly prevalent country in terms of HBV infection, where around nine million people are already infected with HBV and the infection rate is increasing. These higher rates of infection can be assigned to poor health facilities, economical status and lack of information or awareness regarding some of the major transmissible infectious agents such as HCV, HIV and HBV (Ali *et al.* 2011). This study was aimed to investigate the association of host genetic polymorphism in PNPLA3, TM6SF2 and MBOAT7 genes and their possible outcomes in steatosis vs non-steatosis patients and chronic HBV infected patient's vs recovered healthy patients. We observed in this study that the ratio of male patients were high 70% as compared to female 30%, possibly due to increase exposure of male to major risk factors. Participants belonged to age group 20-29 have high prevalence rates of 37%, which are probably young workers. Different studied have reported various age groups of individuals in terms of HBV infection like a study carried out in Karachi revealed different rates of prevalence of HBV in age groups, similarly Khalid and Ghias concluded that increase in age of the patient by one year can result in upsurge in the disease risk by 1.034 times (Ali *et al.* 2015).

The highest percentage of people used Municipal water 65% for drinking. Followed by 32% of individuals who utilized Well water as a source of drinking. Water source do not have any direct implication in transmission of HBV infection, no evidence of faeco-oral route transmission, but hygienic condition of the area does matters. We demonstrated the symptoms in clearance and chronic HBV infected patients. The most obvious symptoms observed in infected patients were abdominal discomfort, fatigue, anorexia and malaise respectively having the rates of 79%, 73%, 65%, and 62%. Female patients followed by fever and abdominal discomfort. Although other symptoms and sign observed in this study like fever, jaundice, dark urine and hepatosplenomegaly were not much higher. Studies have reported different symptoms associated with HBV infection like a study

reported that the most common symptom associated with HBV infection were abdominal discomfort and fatigue, which is in concordance with our study (Ray 2017; Kumar *et al.* 2017). Symptoms were distributed equally among male and female patients. Whereas hepatomegaly was found to be most prevalent sign in steatosis as compared to Non-steatosis, 100% vs 0%, the variation is may be due to fatty liver.

Demography of the patients revealed that large influx of patients was from Peshawar 37%, and followed by Khyber Agency 21%. This high infection rate, probably due to the fact that Peshawar is densely populated area in KPK region. While people living in Khyber Agency are mostly single ethnic background and infrastructure development is relatively poor. We further scrutinized various risk factors associated with HBV infection in studied cohort. The most leading risk factor among HBV patients was injection use 87% followed by intravenous infusions, 83%. Barber visits 70% and contact with jaundice person 43% were also the most frequent risk factors in the studied individuals. Although skin piercing, beauty parlor visit in females and barber visits in males were the most frequent risk factors in both gender, which were traced out significantly different between both genders. Studies have reported different risk factors associated with HBV infection, like a study conducted in Karachi Pakistan have reported that injection were the main risk of HBV 19% and HCV 44% infection (Khan *et al.* 2000).

Similarly another study also reported that injections use was major risk of acute HBV 53% infection (Usman *et al.* 2003). Other studies by different authors have found different results from diverse areas of Pakistan (Jafri *et al.* 2006; Shazi and Abbas *2006*). Another study in Islamabad Pakistan found that contaminated razors could contribute 13% of viral hepatitis transmission (Janjua and Nizamy 2004). In this study risk factors among male and female showed probability value (p) of <0.021, and the association was found significant. We also reported another important risk factor contact with jaundice person in 43% of patients, indicates joint family system in the region. While HBV intra-familial spread is understood in other countries (Lobato *et al.* 2006; Abdool *et al.* 1991). In Pakistan, all the volunteer and replacement donors are screened for viral infection in countryside areas, the estimated prevalence was high. This may be due to poor

infrastructure and poor health facilities in such areas. Additionally the protocol of blood transfusion was compromised in respective areas.

As stated earlier that different factors such as environmental, viral and host genetic factors can really determine the outcomes of HBV infection. HBV factors that can interplay abeyant role in deciding the outcomes of infection include HBV genotypes, DNA levels, variants and co-infection with different viruses. Studies have shown that higher HBV DNA levels are targets for T cells and lower its efficacy. Thus results in the development of hepatocellular carcinoma (Ohkubo *et al.* 2002; Yu *et al.* 2005). Studies have shown that treatment of HBV infection with lamivudine in combination with interferon has resulted in reduction of HBV viral load, similarly it has been shown that HBV genotype B is involved in the severe development of HCC (Kao *et al.*2000; Ding *et al.*2001).

Host genetic factors and their possible outcome in terms of HBV infection are less understood. Many studies have reported contradictory finding in relation to these genetic variants, but it might be due some other reasons, like ethnicity of patients, sample size, or different study designs and study protocol (Vanwolleghem *et al.* 2015). Different immune system pathways are involved in determining the final fate of HBV infection in patients, like JAK-STAT pathway. This pathway is known to be involved in HBV clearance, however studies have also shown that this pathway has also a particular role in liver inflammation, therefore it is very necessary to know the host genetic immunogenetic associations and their role in disease outcomes (Stattermayer *et al.* 2014).

Single nucleotide polymorphisms in the immune system genes play a crucial role in the disease or therapy outcome, such as SNP rs7574865 in the intron of *STAT4* gene is known to have a role in response to IFN therapy and spontaneous clearance of HBV. Similarly, GG genotype at this SNP is known to have a role in increased risk to HCC in Asian population, however the exact mechanism of this particular SNP is still unknown in other populations (Jiang *et al.* 2013). In addition, we also don't understand that how this particular SNP is playing an important role in the pathogenesis of the disease and therapy outcomes. In current study we reported that minor allele (T) of

*STAT4*rs7574865significantly associated with HBV persistence (p=0.04) and also assessing it by recessive model (p=0.02). In contrast, the minor allele of *IFNL3* rs12979860 was not significantly different between HBV-infected patients and clearance control (p=0.3). We also assessed the association of rs7574865 and rs12979860 in steatosis and Non-steatosis patients, but no significant difference was found.

Studies in Chinese population have revealed that rs7574865 GG genotypes has been a predictor of development of chronic infection(Lu *et al.* 2015) however our data also suggest that this particular genotype is also involved in chronic HBV persistence in studied group of patients. The exact mechanism which contributes in the persistence of infection for this SNP is still unknown. The rs7574865 may play a role in changing the expression of transcription binding site or it may affect the splicing of the gene. The second mechanism could be that this SNP is in linkage disequilibrium (LD) with other alleged SNPs and plays an important role in the phosphorylation of STAT4, however the exact mechanism is yet to be discovered.

Genotype rs12979860 of IFNL3 gene is known to be involved in inflammation of liver, however there is an uncertainty about the rs12979860 genotype developing chronic hepatitis and inflammation (Bochud *et al.* 2012; Marabita *et al.* 2011). Some studies have reported different findings to these genetic variants. Likely a report has suggested that CC genotype for the above mentioned SNP can result in worse liver necrosis and less fibrosis and not related with progression to fibrosis hence worse outcomes as compared to /CCTT genotype based on the analysis of the paired biopsies (Noureddin *et al.* 2015). These results are difficult to reset as we know that liver fibrosis is the result of inflammation (Poynard *et al. 2001*) in liver and biopsies suggest that necro-inflammation can best show the level of progression to fibrosis (Ghany *et al. 2003;* Ryder *et al. 2004*).There are some meta-analysis studies carried out in this context and they have shown that rs8099917 TT and rs12979860 CC genotypes were not related with high grade inflammation of liver in patients who had not undergone any sort of therapy, similarly it was also reported that rs12979860 CC genotype is weakly related to fibrosis progression (Sato *et al.2014*).

This study further carried out the comparison of the presentation of various clinical parameters in both the SNP genotypes and they found no significant association among the studied genotypes. Contradictory results have been shown by various studies as they reported the relative association among rs12979860 and rs8099917 genotypes and hepatic histology (Marco *et al. 2012;* Fabris *et al. 2011*). The consequences of rs12979860 genotypes on the liver fibrosis are difficult to explain whereas the above mentioned study confirms its role in inflammation of liver (Thompson *et al. 2013;* Petta *et al. 2012*). There are some studies that have reported no relationship or minor relatedness among the rs12979860 genotype TT (or rs8099917 GG) with liver fibrosis or cirrhosis (Noureddin *et al.* 2015; Falleti *et al.* 2011).

Host genetic factors and their possible outcome in terms of HBV infection are less understood. It is the first report that elucidates the role of two-GWAS based polymorphisms, namely *STAT4* and *IFNL3* in the risk of HBV persistence in the population of KPK region of Pakistan. In studied cohort, we established that *STAT4* but not *IFNL3* is associated with HBV persistence. The observed association was independent of other known variables related to HBV persistence. Polymorphism in *STAT4* rs7574865 was newly reported by GWAS as a risk hotspot for HCC. Subsequent studies suggest that the same polymorphism is related with the whole plethora of HBV infection counting risk of HBV persistence, response to therapy response and progression to cirrhosis in Chinese population (Jiang *et al. 2016;* Lu *et al.* 2015).

This study reiterates that the same polymorphism is also a predisposing factor for HBV persistence in Pakistani populations. Though the underlying molecular mechanisms associated with *STAT4* rs7574865 regarding HBV persistence are yet to be completely understood, some hypotheses could be postulated. *STAT4* rs7574865 risk allele has been shown to be correlated with low *STAT4* mRNA and protein expression in immune cells that is likely to be associated with attenuated function of stimulated natural killer (NK) cells that may lead to the impaired ability of viral clearance by the host (Lamana *et al.* 2015). However, exact role of *STAT4* polymorphism awaits further investigation.

Another intriguing finding from our study, that *IFNL3* polymorphisms, that has been shown by numerous studies to be the strongest single genetic predictor of spontaneous and treatment induced clearance of HCV are not associated with HBV clearance in this cohort. Indeed, the role of *IFNL3* polymorphisms in HBV infection is less clear compared to HCV. Other than this study, previously other reports failed to discern a role of *IFNL3* polymorphisms in HBV clearance or response to therapy (Takahashi 2014). This is likely due to the differential immune response involved in HCV and HBV diseases (Eslam *et al.* 2017; Asimakopoulos *et al. 2016*). It is noteworthy, that a significant correlation between *IFNL3* or *STAT4* polymorphisms with respective to some of the studied variables including HBV-DNA, cholesterol, Platelets, ALT and AST was also observed.

In conclusion, this study establishes that *STAT4* but not *IFNL3* polymorphisms are associated with HBV persistence in population of KPK region in Pakistan. Further studies will be required to clarify the underlying molecular and cellular mechanisms of this genetic association and the differential immune response between the two common viral hepatitis B and C.

NAFLD is comprised of a range of conditions, categorized using grading the vesicular steatosis of liver cells specifically in the lack of substantial alcohol drinking. NAFLD is one of the most prevalent liver disease nowadays, as studies show that this liver abnormality affects more than 1 billion people globally (Loomba *et al.* 2013). This disease is prevalent in developed and developing countries, as prevalence rates of between 3% to 24% are shown for most of the affected countries. Several reports from China have revealed that changes in dietary behaviours and life style have resulted in higher levels of NAFLD in the population (Amarapurkar *et al.* 2007). Interestingly around 30% of the NAFLD patients can progress to the development of NASH, which is one of the aggressive liver abnormalities such as ESLD and HCC (Fan *et al.* 2005).

In addition, genetic and ethnic alterations can also have a prominent role in the presence of NAFLD in a particular population. It has been reported that NAFLD is more likely to develop in East Asian Indians, preceded by Caucasians, Hispanics and African

Americans (Adams *et al.* 2005; Petersen *et al.* 2006). In this study we analyzed the association of MBOAT7 gene SNP with chronic HBV infection and liver steatosis and we found that SNP in MBOAT7 gene were not associated with chronic HBV infection and hepatic steatosis. In addition, we also conducted a combinatorial analysis to find out that SNPs in MBOAT7, PNPLA3 and TM6SF2 can affect the steatosis and. Role of PNPLA3 in the various disease outcomes has been studied thoroughly, however we investigated whether the role of this gene is affected by the presence of TM6SF2 or MBOAT7 alleles. Liver cells are responsible for the excretion of very-low density lipoprotein and it is affected by TM6SF2 gene. Changes in the activity of TM6SF2 gene can result in high levels of triglycerides and hence may lead to NAFLD (Seko *et al.* 2018; Mahdessian *et al.* 2014).

SNPs in TM6SF2 gene are known to be associated with hepatic abnormalities. Genetic variations within TM6SF2 gene have been linked with a number of liver conditions; however, reported results are varying. TM6SF2 rs58542926 was identified as a modifier of hepatic fibro-genesis (Kozlitina *et al.* 2014) and was associated with histological severity of steatosis, increased hepatic inflammation and fibrosis (Liu *et al.* 2014; Dongiovanni *et al.* 2015). Similarly, study conducted by Coppola et al showed that TM6SF2 rs58542926 genotype is associated with progression to lethal steatosis in patients that are co-infected with hepatitis C infection, however there was no evidence found among the TM6SF2 genotype and hepatic inflammation (Koo *et al.* 2018).

Interestingly the PNPLA*3* genotype is known to be modulating the outcomes by affecting development into steatosis and fibrosis. Similarly reports have also shown that *MBOAT7* genotypes are also involved in damaging the liver by scaring (Coppola *et al.* 2015). Our study also confirms the observations made by early reports regarding NAFLD (Krawczyk *et al.* 2017). It is a well-established fact that patients having *PNPLA3*-associated steato-hepatitis (PASH) are already in risk as they are recognized to be at jeopardy of more severe liver fibrosis and HCC (Sookoin *et al.* 2011). However, there are some in-consistent reports regarding *TM6SF2* genotypes and liver diseases.

Our study is one of the primitive studies reporting the relatedness among *MBOAT7*, *MBOAT7* and *PNPLA3*genotypes and their possible role in the development of steatosis and chronic HBV persistence. Recent reports have shown PNPLA3 I148M polymorphism as one of the main factor for fat build-up and liver inflammation due to steatosis (Dongioyanni *et al.* 2014; Sookoian and Pirola *et al.* 2011). The key point in the findings of this study is that it elaborates the *PNPLA3* rs738409, *TM6SF2* rs58542926 genotypes association with development of steatosis in chronically infected HBV patients. Our study shows that *PNPLA3* rs738409, *TM6SF2* rs58542926 SNP is a predictor of chronic HBV infection along with a factor is steatosis development. Minor allele frequency (MAF) for G variant in *PNPLA3* rs738409 had been (p=0.031), significantly different in healthy and HBV patients. We further observed rs738409 GG genotype in 3% of patients, was associated with steatosis (p=0.033). Similarly, *TM6SF2* rs58542926 MAF has also been shown to be significantly associated with steatosis (p=0.024).

A study conducted in Asian population revealed that SNP I148M in PNPLA3 gene in individuals who drink tea and coffee were related to the development of steatosis in analysed individuals, however quantitative relationship between coffee and tea drinking was not analysed (Valenti *et al.* 2012). Eslam *et al* reported that rs58542926 T variant was involved in the development of steatosis in Chinese patients who were chronically infected by HBV (Peng *et al.* 2012). In-addition studies have reported that homozygosity for p.148M variant may result in predisposition to the development of hepatocellular carcinoma, when matched with homozygotes at p.I148. These investigations were carried out and confirmed in individuals having NAFLD (Eslam *et al.* 2016; Burza *et al.* 2012). In our study we found that TM6SF2 T allele resulted in conferring risk for liver steatosis in all groups and this conclusion was made using regression analysis (Odds ratio (OR) 0.2, 95% CI 0.07 – 0.89, p= 0.032).

There are some studies who have reported that TM6SF2 rs58542926 C-allele is greatly related to increased cholesterol whereas T allele is involved in upregulating the lipid retaining by the liver (Krawczyk *et al.* 2015). The rigorousness of non-alcoholic steatohepatitis and non-alcoholic fatty liver disease was not associated with E167K

genotype, there was a liberated result of the mentioned SNP and liver fibrosis (Holmen *et al.* 2014). In contrast a study reported that the above-mentioned variant was associated with increased chances of developing NASH in patients who were identified by hepatic biopsy (Liu*et al.*2014). These gathered reports have revealed an overwhelming association among E167K and development of non-alcoholic fatty liver disease, however the exact role of TM6SF2 E167K SNP is still unknown in development of NAFLD (Dongioyanni *et al.* 2015).

In contrast, the genotype distribution of *MBOAT* rs641738 in this study was not significantly different between HBV-infected patients and healthy control (p=0.137) and also using the recessive model (p=0.500). Similarly, distribution of *MBOAT* rs641738 among steatosis and control group was not significantly different (p=0.666) and also using the recessive model (p=0.448). In a nutshell this study finds out that *MBOAT* rs641738 genotype was not significantly associated with HBV persistence and hepatic steatosis. Similarly, no association was found in MAF for *MBOAT* rs641738. MBOAT rs641738genetic polymorphism is well understood in European population having NAFLD, however it was observed that this particular variant is not related to liver steatosis. Later on it was confirmed that the above mentioned variant was related to liver fibrosis (Chen *et al.* 2017; Mancina *et al.* 2016).

Studies conducted in Asian population revealed that this variant did not posed any risks for mounting effects of NAFLD (Krawczyk *et al.* 2017). Interestingly there are some studies which found a relationship between liver inflammation and rs641738 but didn't report any relationship with steatosis (Koo *et al.* 2018). Nonetheless there are no significant reports confirming the importance of MBOAT variant with liver fibrosis of varying etiology. Our study reports that MBOAT7 SNP rs641738 did not showed any association with hepatic steato-hepatitis.

A combined analysis of MBOAT7 rs641738 and PNPLA3 rs738409 SNP also did not revealed any significant association. PNPLA3 rs738409 CG genotype was found in participants having young age as compared to those with CC and GG genotype (p=0.009). Similarly, the prevalence of T allele reflected higher ALT level, whereas T

allele in MBOAT7 was involved in low triglyceride level. Liver enzymes such as ALT/AST can be regarded as possible indicator of hepatic damage and NASH. Studies have confirmed the association of TM6SF2 genotypes and higher levels of liver markers from different cohorts (Thabet *et al.* 2016). Our analysis indicated no significant relationship among MBOAT7, PNPLA3 and TM6SF2 genotypes with the HBV DNA viral load. Similar works have been reported by various studies as they show that in model organisms studies the HCV RNA levels is usually associated confidently, whereas it is opposite for HBV DNA, as they relate inversely in respective to serum triglycerides (Kozlitina *et al.* 2014; Khattab *et al.* 2012).

We also demonstrated the association of HBV DNA level with clinical variables, and we find out that HBV DNA level is directly proportional to serum cholesterol (p=0.044) and triglyceride (p=0.010). Our study indicate a very significant perspective of clinical importance TM6SF2 must be deliberated with PNPLA3 in describing relationship among TM6SF2 genotypes and NAFLD (Chiang *et al.* 2013; Romeo *et al.* 2008). This study reports a number of genes that are involved in the liver lipid acquisition and persistent HBV infection. Interestingly odds ratio for steatosis represented by TM6SF2 SNP was either same or two times greater than the observed and has been immensely studied PNPLA3 rs738409 variant (Valenti *et al.* 2010; Valenti *et al.* 2010).

In this study, we investigated for the first time the role of functional polymorphisms on three main lipid related genes, namely *PNPLA3*, *TM6SF2* and *MBOAT7* in hepatic steatosis and HBV chronicity in Pakistani population. Consistent with other data reported in different population, we demonstrated that *PNPLA3, TM6SF2* but not *MBOAT7* are related to hepatic steatosis in HBV patients (Thabet *et al.* 2017; Vigano *et al.* 2013). Interestingly, our data suggest these two polymorphisms may be implicated in HBV persistence. The natural history of viral hepatitis infection including HBV infection exhibited marked inter-individual variation, indicating a pivotal role for genetic basis in shaping the outcome of those patients (Eslam and George 2015) The last years have witnessed multiple GWAS studies, which revealed multiple risk variants for common complex diseases including infectious diseases. (Romeo *et al.* 2008; Eslam *et al.* 2017) However, to date, most of the studies were conducted in either Caucasian or other Asian

population, with very limited data in Pakistani population, a country having very great incidence of HBV infection in large population.

Patients with hepatic steatosis tend to have higher age and serum cholesterol and triglyceride, though it was not significant, but no difference was noticed in HBV-DNA levels. Consistent, with what have been suggested that steatosis in HBV is metabolic rather than viral. In this context, a polymorphism in the *PNPLA3* gene, which is known as adiponutrin and has triacylglycerol lipase activity have been shown robustly by multiple studies to have a strong association, with liver fat content in NAFLD as well as in patients with viral diseases (Pirazzi *et al.* 2014; Trepo *et al.* 2016). Similarly, nonsynonymous coding variants in the *TM6SF2* (E167K) are analyzed greatly as a predisposing factor for hepatic lipid buildup and advancement to NASH.

Similar data was reported with hepatic steatosis in patients with viral hepatitis. (Kozlitina *et al.* 2014; Milano *et al.* 2016) However, the exact function of *TM6SF2* is not known, but it regulates cholesterol synthesis and the secretion of lipoproteins (Mahdessian *et al.* 2014). This polymorphism in the *TM6SF2* gene (rs58542926) impacts hepatic *TM6SF2* mRNA and protein expression.

In contrast, the association between polymorphisms in the *MBOAT7* and hepatic steatosis is controversial, with most of the evidence suggest the lack of the association or at the best it would be very marginal. Though, the mechanisms of *MBOAT7* functions are still unclear, it is likely involved in hepatic inflammation, via its function in the remodeling pathway of phosphoinositides (Land's cycle) that attaches arachidonic acids (AAs) to lysophosphatidylinositol and also could augment the inflammatory reaction in macrophages and supplementary immune system cells (Mancina *et al.* 2019; Thabet *et al.* 2016)

Viral hepatitis including HBV has an intimate interaction with lipids (Khattab *et al.* 2012; Li *et al.* 2015). Our data suggest that polymorphisms in *PNPLA3* and *TM6SF2* but not *MBOAT7* might be implicated in HBV persistence. This consistent with a couple of reports suggest these two polymorphisms are modulating HBV-DNA (Romeo *et al.* 2008; Khattab *et al.* 2012). Furthermore, the lack of association between *MBOAT7* and HBV

persistence is consistent with our current data and others that indicate that the consequences of *MBOAT7* on hepatic diseases are doubtful regarding hepatic steatosis. More analysis is needed to comprehend the functional mechanisms of these effects and if it can be exploited for therapeutic purposes.

Conclusion

In conclusion, this is the first report that highlights HBV/HDV infection in outpatient department patients, from KPK region, which indicates higher prevalence of HBV/HDV infection in patients with no prior history of any kind of hepatitis and majority of these patients, belong to younger age group. In addition, serological and viral DNA based evidence suggest active prevalence of HBV infection among majority of the patients which indicates higher risk of further HBV transmission by these patients. Indispensable efforts are required in order to control the co-infection of HBV/HDV in the population of KPK in Pakistan. Similarly, the second part of the study establishes that *STAT4* but not *IFNL3* polymorphisms are associated with HBV persistence in population of KPK region in Pakistan. Further studies will be required to clarify the underlying molecular and cellular mechanisms of this genetic association and the differential immune response between the two common viral hepatitis B and C. The third portion of the study reflects the association of polymorphisms in *PNPLA3* and *TM6SF2* with steatosis and HBV viral healthy in Pakistani population. In addition the same analysis concludes that polymorphism in the *MBOAT7* gene is not related to steatosis or persistence of HBV infection in Pakistani population. Future direction will be to explore if these findings can aid in guiding efforts for personalization of medicine and finding novel therapeutic targets.

Future prospect

- To establish the association of other host immune genetic factors like TNF HLA-DP/DQ, TGFβ1, *GCKR* and IL-6 along with these analyzed SNPs in HBV therapy or disease outcomes.
- To investigate the role of *STAT4*, *IFNL3PNPLA3*, *TM6SF2* and *MBOAT7*genetic polymorphisms in response to interferon and lamivudine therapy in chronic hepatitis B patients.
- To generate knock out mouse models in order to confirm theses variants and their respective association among the studied group of patients.

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PNPLA3 and *TM6SF2*, but Not *MBOAT7*, Are Associated with Steatosis and HBV Viral Persistence in Pakistani Population

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Abstract

Background: Hepatitis B infection has an intimate relationship with lipids. The role of lipid-related variants remains unknown in the risk of hepatitis B infection persistence and steatosis in the Pakistani population. Recently, three GWAS-based polymorphisms in the *TM6SF2*, *PNPLA3*, and *MBOAT7* genes have suggested being associated with steatosis and/or liver injury. However, the role of these variants is unknown in Hepatitis B virus (HBV) persistence and steatosis in the Pakistani population.

Objectives: We determined whether *TM6SF2*, *PNPLA3*, and *MBOAT7* genetic variations are associated with HBV chronicity and hepatic steatosis in the Pakistani population.

Methods: A total of 297 patients visiting the Hayat Abad Medical Complex in Peshawar were included in this study. Clinical analysis, along with genotyping of SNPs in the *PNPLA3*, *TM6SF2*, and *MBOAT* genes, was performed using the TaqMan genotyping assay. Logistic regression analysis, along with other tests as appropriate, was used to determine the association of the analyzed SNPs with HBV persistence, chronicity, and hepatic steatosis in the analyzed set of patients.

Results: In 297 subjects (240 HBV patients and 57 healthy controls), *PNPLA3* rs738409 (OR: 0.43, 95% CI: 0.23 - 0.81, P = 0.009) and *TM6SF2* rs58542926 (P = 0.018) genotypes were independently associated with the risk of chronic HBV infection, but not *MBOAT* rs641738 (OR: 1.3, 95% CI: 0.64 - 2.62, P = 0.454). We also observed that the *PNPLA3* rs738409 GG genotype was associated with 2.97-fold and *TM6SF2* rs58542926 genotype T allele with 1.54-fold increased risk of steatosis.

Conclusions: *PNPLA3* rs738409 and *TM6SF2* rs58542926, but not *MBOAT* rs641738, were the risk variants for HBV persistence and steatosis in the Pakistani population.

Keywords: CHB, *TM6SF2*, *PNPLA3*, *MBOAT7*, Steatosis, Persistence

1. Background

Hepatitis B virus (HBV) is highly prevalent, as one-third of the world's population has been exposed to it and 350 - 400 million have developed a chronic infection, with > 1 million deaths per year from cirrhosis and liver cancer (1). In Pakistan, almost more than 9 to 12 million people are estimated to be living with HBV or Hepatitis C virus (HCV), with a carrier rate of 3% - 5% (2, 3). Hepatitis B virus is responsible for more than half of all new cases of liver cancer and it is among the "top ten" causes of cancer death. On the other hand, non-alcoholic fatty liver disease (NAFLD) is currently on a trajectory to become the most common liver disease, as it has affected around 20 - 30% of the global population (4). This renders that the co-occurrence of both diseases is not infrequent and studying this relationship is of pivotal importance.

Hepatitis B has an intimate link with hepatic lipid metabolism. An opposite association is observed between positive Hepatitis B surface antigen (HBsAg) status and the prevalence of fatty liver in humans. It has been suggested that HBsAg seroclearance is more than three-fold higher in those with moderate-to-severe hepatic steatosis than in those without hepatic steatosis (5). A similar observation was reported in mouse models and *in vitro* (6). Multiple reports have identified variants in patatin-like phospholipase domain containing 3 (*PNPLA3*) (I148M) and transmembrane 6 superfamily member 2 (*TM6SF2*) (E167K) as risk variants for steatosis in patients with NAFLD. However, the data are scarce on the impact of these variants on hepatic steatosis in patients with viral hepatitis, especially in Asian patients with hepatitis B. Notably, it is proposed that these polymorphisms are associated with HBV-DNA levels, sug-

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Original article

The *STAT4* and not the *IFNL3* variant is associated with hepatitis B virus clearance in a population from the Khyber Pakhtunkhwa region of Pakistan

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ABSTRACT

Background and study aims: Host genetic modifiers of the risk and persistence of hepatitis B virus (HBV) infection in the Pakistani population have not been clearly elucidated. Recently, two genome-wide association studies described that *STAT4* and *IFNL3* variants are associated with different aspects of the course of HBV infection. However, the roles of these variants in the persistence of HBV infection have not been investigated in the HBV-infected population of Pakistan. Therefore, we examined the roles of the *STAT4* and *IFNL3* variants in a chronic HBV-infected population from the Khyber Pakhtunkhwa (KPK) region of Pakistan.

Patients and methods: *STAT4* rs7574865 and *IFNL3* rs12979860 genotyping were performed in 297 subjects (240 infected with HBV and 57 controls). Statistical analyses were performed using the chi-squared test, Students *t*-test, HardyWeinberg equilibrium tests and logistic regression models.

Results: Among the 297 subjects, compared with the *IFNL3* rs12979860 genotype [odds ratio (OR) = 0.7, 95% confidence interval (CI) = 0.391.29, $p = 0.2$), the *STAT4* rs7574865 genotype was independently associated with the risk of developing chronic HBV infection [OR = 1.9, 95% CI = 1.093.50, $p = 0.02$].

Conclusion: The *STAT4* rs7574865 and not the *IFNL3* rs12979860 variant is associated with persistence of HBV infection in a Pakistani population from the KPK region.

Introduction

One-third of the global population has been exposed to hepatitis B virus (HBV), and approximately 350400 million patients develop chronic HBV infection annually. In addition, cirrhosis and liver cancer alone are responsible for >1 million deaths every year [1]. Pakistan is one of the countries with the highest prevalence rates of HBV infection in the world, with more than 9 million individuals living with the infection [2,3].

The course and outcome of HBV infection are determined by complex and dynamic interactions among immune responses and viral and host genetic factors [2]. Recent genome-wide association studies (GWAS) have enhanced our understanding of the genetic factors influencing the entire course of viral hepatitis [4,5]. As highlighted recently, the host genetic basis is a crucial contributor to the augmentation of the risk of HBV persistence [6]. Similarly, recent GWAS have established that the single-nucleotide polymorphism (SNP) rs7574865 in *STAT4* is associated with the risk of developing hepatocellular carcinoma (HCC). Subsequent studies have conceived that the same polymorphism is implicated in the full spectrum of HBV infection, including the risk of viral persistence, response to interferon-based therapy and progression to significant fibrosis and cirrhosis [7,11].

Furthermore, multiple landmark GWAS have recently established the role of the *IFNL3/IFNL4* variant in hepatitis C virus (HCV) infection and in both spontaneous and treatment-induced clearance [12,14]. These variants are reportedly associated with the risk of developing liver fibrosis, HCC [4,15] and organ fibrosis [16]. However, the role of the *IFNL3/IFNL4* variant in HBV infection remains largely unexplored in various populations, including the HBV-infected population in Pakistan. Moreover, the allelic frequency of polymorphisms, the haplotype distribution and the effect size vary significantly across different ethnicities and populations. In this context, a recent study suggested that *STAT4* rs7574865 is related neither to the response to interferon therapy nor to spontaneous clearance in Caucasian-origin patients with

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Seroprevalence of HDV among non-hospitalized HBsAg positive patients from KPK-region of Pakistan

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ABSTRACT

Objective: To study the seroprevalence of hepatitis B virus (HBV) and hepatitis delta virus (HDV) infections in patients visiting outpatient department of a major tertiary care hospital in Khyber Pakhtunkhwa region of Pakistan.

Methods: Blood samples were collected from non-hospitalized patients. Serological analysis was done by ELISA and viral DNA was amplified by PCR. The amplified DNA was analyzed by agarose gel electrophoresis.

Results: Altogether, 946 blood samples were screened, overall percentage of HBsAg-positive patients remained 22.41% (prevalence: 224.10/1 000; *CI*: 0.197 5 ± 0.250 7) with the highest incidence rates among relatively younger age groups (20–29 years). The prevalence of HBV–HDV co-infection was found to be 46.75/1 000; *CI*: 0.031 8 ± 0.061 7. In HBsAg-positive patients, anti-HBc-total was detected in 86.79% while 25.00% were positive for anti-HBc-immunoglobulin M. Similarly, among these patients, HBV DNA was detected in 64.13% and 10.85% were co-infected with HDV. Different symptoms were associated with the prevailing infection, including malaise (62%), anorexia (66%) and fatigue (73%). The most commonly associated symptom was abdominal discomfort. Among these patients, certain risk factors, including surgery, visit to dentist and intravenus infusions were frequently associated with the infection ($\chi^2 = 95.23$; $df = 11$; $P < 0.000\,1$).

Conclusions: Overall, this study confirmed higher prevalence of active HBV/HDV infection, among young patients from Khyber Pakhtunkhwa region having no prior history of viral hepatitis.

1. Introduction

Hepatitis delta virus (HDV) is a defective negative-sense RNA virus that requires hepatitis B virus (HBV) for its assembly [1]. The genome of HDV is circular and consists of 1 700 nucleotides in length [2]. Both HBV and HDV have the same route of transmission [3]. It is estimated that out of 350 million HBV carriers, 18 million people are co-infected with HDV [4]. Co/super-infections due to HDV leads to severe acute liver disease and increases the risk of developing fulminant hepatitis [5]. Different studies conducted around the globe reported varying prevalence rates of HDV. For example, in Middle-East prevalence rate was reported to be 3.3% among the general population, while particularly in Saudi-Arabia, it was 8.6% in the hospitalized patients. In Oman, up to 7.7% of the registered dialysis patients were infected with HDV [6,7]. Similarly, from China prevalence rates were reported to be 7.2% in Shandong and 3.5% in Henan provinces [8].

From Pakistan different prevalence rates for HDV infection have been reported, ranging from 16.5% to 58.6% [9,10]. However, with the exception of few reports, most of these studies focused on stratified groups like hepatitis patients or patients at higher risk of HDV infection. Hence, little is known about the prevalence of

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The study protocol was performed according to the Helsinki declaration and approved by institutional research and ethics board of Hayatabad Medical Complex. A formal consent was obtained from each patient or his/her guardian/parent.

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