2020-01-06

Characterization of Hepatitis B Virus (HBV) and Host Cytokine Patterns in a Multiethnic Cohort of Patients with Non-alcoholic Fatty Liver Disease (NAFLD) and Chronic Hepatitis B (CHB)

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Characterization of Hepatitis B Virus (HBV) and Host Cytokine Patterns in a Multiethnic Cohort of Patients with Non-alcoholic Fatty Liver Disease (NAFLD) and Chronic Hepatitis B (CHB)

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

GRADUATE PROGRAM IN MICROBIOLOGY AND INFECTIOUS DISEASES

CALGARY, ALBERTA

JANUARY, 2020

Abstract

Studies have reported conflicting data on the relationship between non-alcoholic fatty liver disease (NAFLD) and chronic hepatitis B (CHB). We aimed to identify how metabolic factors associated with NAFLD (diabetes, hypertension, central obesity and dyslipidemia) affects the hepatitis B virus (HBV) in patients with CHB.

Patients with CHB and NAFLD were prospectively enrolled from 3 Canadian liver clinics. Patients underwent standardized liver tests (liver stiffness measurement [LSM] by transient elastography, controlled attenuation parameter [CAP]) and HBV clinical tests (quantitative [q] HBV surface antigen [HBsAg], HBeAg). Plasma levels of HBV DNA and RNA were measured by quantitative (q)PCR. Viral genotype was identified by population and next generation sequencing of the precore (C)/C and presurface (S)/S genes and analyzed using MEGA 7. Peripheral blood mononuclear cells (PBMCs) were stimulated *ex vivo* for 72h by HBV core antigen (HBcAg) or HBsAg peptides. PBMC supernatant and serum were analyzed for cytokine/chemokine markers using a 13-plex immunoassay. Kruskal-Wallis, multiple linear regression, Chi-square, and Fischer's exact tests were performed using R commander.

Of 48 subjects enrolled (median age 44.5 [IQR 16.8]), most were male (n=31), of Asian descent (n=29), and HBeAg negative (n=45). In HBeAg negative patients, the mean CAP was 306±52 dB/m, ALT was 40±26 IU/mL, and LSM was 5.8±2.0 kPa, indicating high steatosis without fibrosis. In all patients, the HBV genotypes were 13% A, 16% B, 46% C, 17% D, 6% E. Mutations associated with severe liver disease, anti-viral drug resistance, immune escape, and HBeAg negativity were identified in all subjects. Obese patients had increased qHBsAg levels, while diabetic patients had

increased S gene diversity. Hepatic steatosis severity did not relate to viral factors analyzed. *Ex vivo* PBMC responses to HBcAg or HBsAg stimulation were not different to unstimulated controls.

In this study, a multi-ethnic cohort of CHB and NAFLD patients were prospectively evaluated with novel virologic and host immunological markers. We found that metabolic factors associated with NAFLD correlated to inflammatory cytokine levels, viral genetic characteristics, and HBV replication markers. These viral and host factors can influence the risk of liver disease progression in patients with both NAFLD and CHB, warranting further study.

Preface

This thesis is original, unpublished, independent work by the author, A. Lucko, with one exception. Figure 1 was sourced from Sunbul, M. Hepatitis B virus genotypes: Global distribution and clinical importance. *World J. Gastroenterol.* **20**, 5427–5434 (2014). All data received were anonymous and collected under an approved University of Calgary Conjoint Ethics Research Board (CHREB) approved protocol (Ethics ID# REB16-0041). All samples were collected and sample/data analysis was performed under CHREB Ethics ID# REB16-1862. Eligible participants signed informed consent forms and the study was approved at each participating site's local research ethics board.

Acknowledgements

I would like to first and foremost thank my supervisor, Dr. Carla Coffin, who has been a mentor to me throughout the last few years at the University of Calgary, and who has provided unparalleled advice, patience, and guidance in my graduate degree and research career. To the other members of Dr. Coffin's lab, Keith Lau, Drs. Shivali Joshi and Vanessa Meier-Stephenson, and all the other temporary students, thank you all for helping to train me and show me the intricacies of the lab. I would like to thank all those involved with the recruitment of patients and organization of the study, particularly Vince Avati, and Drs. Sarah Haylock-Jacobs, Aziz Shaheen, Karen Doucette, Stephen Congly, Meredith Borman, and Alnoor Ramji. I would also like to thank Drs. Carla Osiowy and Guido van Marle for their invaluable assistance and advice with sequencing protocols and analysis. Without these people, I would not have completed this project and I am extremely grateful and in debt for their assistance. I would also like to thank the Queen Elizabeth II studentship, and University of Calgary for providing graduate studentship funding that were essential to supporting my studies. Lastly, I would like to thank my wife, Caroline Corbett, for her compassion, her advice, her willingness to put up with my strange work habits, and her absolute support throughout my entire degree and career. I could not ask for a better partner to help me through trying times.

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AHB – acute hepatitis B

ALT – alanine aminotransferase

Anti-HBc – HBV core antibodies

Anti-HBe – HBV E antibodies

Anti-HBs – HBV surface antibodies

APOBEC – apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like

BMI – body mass index

C – HBV Core

CAP – controlled attenuation parameter

cccDNA – covalently closed circular DNA

CHB - chronic hepatitis B

ChREBP – carbohydrate response binding-element protein

DNA – deoxyribonucleic acid

EDTA - ethylenediaminetetraacetic acid

ER – endoplasmic reticulum

FABP1 – fatty acid binding protein 1

FBS – fetal bovine syndrome

GM-CSF – granulocyte-macrophage colony stimulating factor

HBeAg –HBV E antigen

HBsAg – HBV surface antigen

HBV - hepatitis B virus

HCC – hepatocellular carcinoma

HIV – human immunodeficiency virus

IFN – interferon

IgG – immunoglobulin G

IgM - immunoglobulin M

JAK - Janus kinase

LB – luria broth

LSM – liver stiffness measurement

MHz – megahertz

MTCT – mother to child transmission

NA – nucleos(t)ide analogues

NAFLD – non-alcoholic fatty liver disease

NASH – non-alcoholic steatohepatitis

NF-kB – nuclear factor kinase-B

NGS – next generation sequencing

NTCP- sodium taurocholate cotransporting peptide

PBMC – peripheral blood mononuclear cells

pgRNA - pregenomic RNA

PHA – phytohemagglutinin

PNPLA3 – patatin-like phospholipase 3

qHBsAg – quantitative hepatitis B surface antigen

RACE – rapid amplification of complementary ends

rcDNA – relaxed circular DNA

S – HBV Surface

SREBP1 – sterol regulatory element-binding protein-1

STAT - signal transducer and activator of transcription protein

Th – T helper cell

TLR – toll-like receptor

TM6SF2 – human transmembrane 6 superfamily 2

TNF – tumour necrosis factor alpha

UPR – unfolded protein response

Chapter 1: Introduction

Hepatitis B is a widespread disease characterized by inflammation of the liver due to infection with the hepatitis B virus (HBV)[1]. Approximately 257 million individuals have serum HBV surface antigen (HBsAg) positive chronic hepatitis B (CHB), with nearly 2 billion people showing serological evidence of exposure to the HBV[1]. There is an effective HBV vaccine to prevent infection, consisting of recombinant yeast produced HBsAg combined with an aluminum hydroxide sulfate adjuvant[2]. Despite introduction of the vaccine in over 200 countries worldwide, more than 800,000 individuals die each year due to hepatitis B, primarily via hepatocellular carcinoma (HCC) and liver failure[1]. In Canada, there are approximately 200,000 individuals living with CHB, with annual costs of treatment ranging from $1,200 to $1,800, without accounting for the costs of monitoring and complications of end-stage liver disease[3,4]. As a result, CHB has a major economic impact on the healthcare system.

Transmission of the HBV occurs through contact with blood or bodily fluids[1,5]. Direct or horizontal contact transmission routes for HBV include sexual and blood contact with contaminated objects such as needles or syringes. The most common route of HBV transmission worldwide is by vertical transmission of the HBV during birth from mother to child[1,5]. Most adults exposed to the virus mount an effective immune response and can successfully clear acute infection. However, individuals infected at birth, without vaccination, have >90% risk of chronic (serum HBsAg positive) infection. Due to the generally high titre of viral particles in blood and structure of the virus, HBV has been shown to survive in the environment for up to 7 days, with viral proteins and genetic material able to survive under harsh conditions, such as freeze/thaw cycles, for

months[6,7]. Once exposed to a contaminated source, HBV is considered to be highly infectious, with estimates that the virus is 100 times more infectious than HIV[8].

1.1 The lifecycle of the hepatitis B virus (HBV): The HBV is an enveloped and double-stranded DNA virus within the *Hepadnaviridae* family[9]. There are 10 HBV genotypes identified, (A-J) that are geographically distributed throughout the world (Figure 1)[10]. Genotypes are separated by at least an 8% difference in nucleotides and have differences in disease outcomes as well as antiviral responses to interferon (IFN) treatment [9,10]. The complete HBV genome is 3.2 kb in length, and codes for 7 proteins over 4 overlapping open reading frames[9]. The viral proteins encoded in the HBV genome are: (1). The viral polymerase (P), which is used to reverse transcribe the HBV genome; (2). The large, medium, and small surface (S) proteins which make up the viral envelope and the secreted subviral free HBsAg spheres or filaments; (3). The core (C) protein, which comprise the viral capsid; (4). The X protein, which affects host cell processes and viral transcription; and, (5). The HBV E-antigen (HBeAg), also encoded by the HBV core gene, which is secreted from the cell and has immunomodulatory functions. Due to the viral reverse transcriptase, the replication cycle of HBV is highly error prone leading to significant viral diversity within an infected individual[9,10]. This diversity can lead to mutations which are associated with altered viral phenotypes, such as immune escape (i.e., mutations in the preS/S gene of proline to leucine or threonine at position 127 [P127L/T], methionine to leucine, isoleucine, or threonine at position 133 [M133L/I/T], threonine to isoleucine at position 140 [T140I], serine to leucine at position 143 [S143L], aspartic acid to arginine or glutamic acid at position 144 [D144A/E],

glutamine to arginine at position 145 [G145R], glutamic acid to aspartic acid or glycine at position 164 [E164D/G], proline to arginine at position 178 [P178R]), HBeAg negativity (i.e., mutations in the preCore (C) gene of guanine to adenine at nucleotides 1866 [G1866A], 1896 [G1896A], or 1899 [G1899A]), anti-viral drug resistance (i.e., mutations in the P gene of phenylalanine to histidine or leucine at position 161 [F161H/L], leucine to phenylalanine at position 173 [L173F], leucine to phenylalanine at position 175 [L175F], leucine to valine at position 176 [L176V], serine to phenylalanine or leucine at position 193 [S193F/L], valine to phenylalanine or serine at position 194 [V194F/S], tryptophan to serine or leucine at position 196 [W196S/L]), as well as worse liver disease outcomes (i.e., mutations in the C gene of phenylalanine to tyrosine at position 24 [F24Y], glutamic acid to aspartic acid at position 64 [E64D], glutamic acid to glutamine at position 77 [E77Q], alanine to isoleucine, valine, or leucine at position 80 [A80I/V/L], or leucine to isoleucine at position 116 [L116I]).

Once an individual is infected by HBV, the virus targets and infects hepatocytes (Figure 2)[9]. HBV entry to hepatocytes is mediated by it's specific cell-surface receptor, sodium taurocholate cotransporting peptide (NTCP), which is primarily a bile acid transporter protein[9]. The host NCTP binds to the preS1 region of the large HBV S protein which triggers the host cell to internalize HBV through endocytosis[9]. The HBV envelope fuses with the endosome, to deposit the viral capsid and its contents, into the cytoplasm[9]. The viral nucleocapsid is then transported to the nuclear pore complex, where the HBV capsid and relaxed circular (rc)DNA are separated during nuclear import[9]. In the viral particle, and during nuclear import, the HBV genome is partially double-stranded and circular (i.e., rcDNA) with an incomplete plus DNA strand. Once

the viral genome is imported into the nucleus, it is converted to a stable episomal intermediate called covalently closed circular DNA (cccDNA) that acts as a template for viral transcription and translation[9]. The HBV cccDNA template generates a full-genome length RNA intermediate known as pregenomic RNA (pgRNA)[9]. The HBV pgRNA also serves as an messenger RNA (mRNA) template for the HBV C and P proteins[9]. Following translation of these two proteins at the host ribosome, the viral P binds to the 5' end of pgRNA[9]. Formation of this ribonucleoprotein complex triggers the formation of the viral capsid, which is comprised of 240 units of C protein enclosing the viral genome[9]. Once the genome is encapsidated, the P is used to reverse-transcribe pgRNA into rcDNA, first by creating the minus-strand DNA through a covalent bond with a hairpin structure in the ribonucleoprotein complex[9]. A plus-strand DNA is then synthesized from the minus-strand using an oligomer derived from the 5' end of the pgRNA as a primer[9]. However, the plus-strand is not a complete complementary strand to the minus and is prematurely terminated[9]. At the 5' end of each of the rcDNA strands are regions with direct repeating (DR) sequences, named DR1 and DR2, which enable the rcDNA to maintain its circular structure[9]. As noted above, the reverse transcription activity of the viral P is highly error-prone, and is the main cause of the high variability of HBV[9]. This high variability means that viral quasispecies or collections of viruses with mutations may form from selective pressures such as those exerted by the host immune system (or antiviral therapy in subjects on treatment)[11]. During DNA synthesis, the viral capsid begins to interact with the small, medium, and large S proteins, which are produced largely in excess of the viral particles[9]. The newly assembled virion, known as the Dane particle, is released from the cell non-lytically via cell secretory systems[9].

Alternatively, newly formed viral nucleocapsids can enter the nucleus to deposit their genetic contents to form a new copy of cccDNA (i.e., cccDNA recycling)[9]. Rather than converting to cccDNA, rcDNA can also integrate into the host genome and will produce viral proteins, such as HBsAg, but is not capable of producing infectious virions.

1.2 The immunopathogenesis of HBV infection: There are two major phases in CHB, primarily marked by E-antigen (HBeAg) positivity. HBV E-antigen was previously used as an indicator of active viral replication, and is often associated with an increased viral load (HBV DNA levels in serum)[12,13]. HBV E-antigen positivity occurs at the beginning of acute hepatitis B (AHB) and CHB[9,14]. CHB patients may eventually seroconvert with HBeAg loss and produce anti-HBe antibodies[15]. However, some viral variants have mutations in the preC/C and basal core promoter regions of the HBV genome that result in reduced expression of the HBeAg[16]. As HBV has a non-cytopathic lifecycle, damage to the liver is primarily a result of the host immune response to the infection[9]. The chronic inflammation leads to an abnormal damage response mechanism that leads to the development and build-up of hepatic scar tissues, or fibrosis. The activation of hepatic stellate cells by inflammatory liver damage leads to their differentiation into myofibroblasts and subsequent cellular proliferation[17]. Myofibroblasts move into the wounded site and secrete a fibrotic extracellular matrix to maintain structural integrity[17]. Once the wound closes the myofibroblasts normally undergo apoptosis, although persistent injury can result in scar tissue formation from myofibroblast persistence[17].

Cirrhosis is the late stage of liver scarring where progressive accumulation of fibrosis impairs liver function[1,5,18]. Liver fibrosis may lead to portal hypertension. Portal hypertension is due to increased vascular resistance and can disrupt the flow of blood in veins leading to the liver[18]. Hepatic blood flow may then be shunted to collateral pathways and lead to development of porto-systemic shunts called varices (dilated submucosal veins and are at risk for rupture and life-threatening bleeding[18,19]. Patients with cirrhosis are also more susceptible to bacterial infections, often through increased microbiome dysbiosis and intestinal permeability leading to infiltration of lymph nodes by bacteria[18]. Bacterial contaminants, such as lipopolysacharrides, and liver damage can increase nitric oxide production. Nitric oxide can stimulate systemic vasodilation, which lowers effective circulating blood volume and activates the renin-angiotensin-aldosterone system. These neurohumoral changes ultimately lead to excess sodium and water retention (i.e., ascites)[18]. Cirrhosis is also a major risk factor for the development of HCC[18].

The initial innate antiviral immune responses to HBV infection primarily consist of toll-like receptor (TLR) activation of immune cells, which are recruited through the production of interferon alpha (IFN-α), IFN beta (β), IFN gamma (γ), and IL-12, and produce inflammatory cytokines such as TNFα[14]. IFNs also activate innate antiviral cellular responses, which affect HBV capsid assembly and induce hypermutations during viral replication through the activation of “apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like” (APOBEC)3G which converts deoxycytidine to deoxyuridine during reverse transcription of the viral genome[20].

The adaptive-immune response to initial HBV infection is slow, and results in the death of large numbers of hepatocytes during clearance of HBV[9,14]. Infected hepatocytes presenting with viral antigens are targeted by T-cell mediated immune responses, with anti-HBV C antibodies (anti-HBc) plateauing between 8 and 24 weeks post-infection, followed by anti-HBc Immunoglobin (Ig)M antibodies being replaced IgG antibodies over time[14]. After approximately 32 weeks post infection, production of neutralizing anti-HBV surface antibodies (anti-HBs) by B cells also begins[14]. Although, T cell mediated immune responses can clear HBV infected liver cells it can lead to active liver damage[14] and is associated with an increase in serum ALT levels. In AHB of adults these immune responses may clear HBV infection; however, in CHB the virus persists and HBsAg/anti-HBs seroconversion does not occur[9,14]. Viral persistence may be due to T-cell dysfunction, which has been noted in CHB patients, and is thought to result from the immune exhaustion of intrahepatic T-cells[15]. Immune exhaustion is the inhibited response to HBV associated antigens by T-cells, and occurs through the constant exposure to high levels of viral antigens (i.e., HBsAg)[15]. Despite this persistence, HBV viral loads may decrease over time in CHB, possibly through antiviral cytokines supressing viral replication or selecting for hepatocytes that do not support high levels of viral replication[9,14].

The immune selective pressure on HBV can result in the selection of viral quasispecies in an attempt to evade detection by the immune system through immunomodulation or immune escape. The HBV can persist as a latent or occult HBV infection (OBI), defined as the presence of replication-competent HBV DNA (i.e. cccDNA) in the liver and/or HBV DNA in the blood of individuals that test negative for

HBsAg by currently available assays. The molecular basis of OBI is unclear but may be due to the epigenetic modulation of HBV cccDNA transcription[21,22].

Intestinal microbiota are known to modulate host immune responses in a wide range of disease, including HBV, via intestinal TLR pathways[23]. Mouse models have demonstrated that clearance of HBsAg is dependent on age-related gut microbiota compositions[24]. Furthermore, this study identified a negative effect of antibiotic clearance of gut microbiota on HBsAg clearance and anti-HBs seroconversion. Another study identified that fecal microbiota transplantation was able to clear HBeAg in patients on long-term entecavir and tenofovir therapies, indicating that the gut microbiota may play a role in modulating host responses to antiviral treatments[25].

1.3. Overview of HBV clinical tests and assessment of liver disease: The gold standard for assessing liver disease is a biopsy, as hepatic HBV cccDNA content, viral antigens, histological inflammation and liver fibrosis can be directly assessed. However, a liver biopsy is invasive, and can result in rare complications and even death[26]. Therefore, non-invasive measures of liver disease progression are used in clinical practise. Liver fibrosis and active liver inflammation can be measured using a liver stiffness measurement (LSM) and ALT, respectively. LSM uses transient elastography via Fibroscan®, which quantifies fibrosis by generating shear waves through the liver at 37 megahertz (MHz), and measuring the attenuation of the waves by the liver[27]. The LSM readings are correlated to a Metavir fibrosis stage, based on prior liver biopsy validation studies. Standard cut-off values for LSM in patients with CHB (without concomitant NAFLD) are 7.2 kPa for moderate fibrosis (Metavir stage 2), 12.5 kPa for

severe fibrosis (Metavir stage 3), and 17.6 kPa for cirrhosis (Metavir stage 4)[28]. Increased serum ALT levels are indicative of active liver damage, as ALT is primarily found within hepatocytes and is released upon cell death[29]. There is evolving evidence on non-invasive serum tests to assess HBV cccDNA activity[13]. As current treatment options for HBV are not curative, treatment is usually limited to patients that are at high risk for liver disease progression[13]. Current end points for HBV treatment is the clearance of HBsAg and seroconversion to anti-HBsAg and is called a “functional cure” (since the intrahepatic HBV cccDNA template can still persist) and is associated with a reduced risk of liver disease progression[13]. Serum levels of quantitative (q)HBsAg and HBV DNA (or viral load), and HBeAg status (Table 1) can be monitored, in combination with other clinical tests, to assess response or determine the need for treatment[13]. Prolonged elevation in HBV DNA levels, particularly in HBeAg negative and older patients, and increased ALT levels may be indicators of liver disease progression[4]. However, HBeAg positive patients, who are usually younger, may have high alanine transaminase (ALT) and HBV DNA may also achieve HBeAg/anti-HBe seroconversion before developing significant liver disease[4]. Recent studies have evaluated the clinical utility of qHBsAg and HBV RNA testing in serum. Monitoring of qHBsAg levels can be used as an indicator of viral transcription of cccDNA and integrated viral DNA, response to treatment, and potential HBsAg seroconversion[4]. Serum HBV RNA can also be used to assess viral activity and are used to identify treatment endpoints, as reduction in RNA is related to an increased likelihood of achieving HBeAg / anti-HBe seroconversion[13].

There are 2 main approved HBV treatment options. Pegylated-IFN, which increases expression of innate inflammatory responses via the Janus kinase (JAK)/

signal transducer and activator of transcription protein (STAT) pathway, can be used for treatment of CHB in certain cases[4]. However, the mode of action of pegylated-IFN is systemic, rather than targeted, and so it is associated with several side effects. Nucleos(t)ide analogues (NAs), such as tenofovir alafenamide, tenofovir disoproxil, and entecavir, are a class of drug that block the reverse transcription action of the viral P[4]. Treatment by NAs reduces serum HBV DNA levels, but require long-term treatment and discontinuation of treatment can lead to ALT flares, as the HBV replication increases[4]. There are also differences in treatment response by HBV genotype, with infections of genotypes C, D, and G showing lower response rates to pegylated-IFN[10].

1.4. The pathogenesis of non-alcoholic fatty liver disease (NAFLD): NAFLD is the most common liver disease in the general population, with up to 20-30% prevalence in the general population[30]. The pathogenesis of NAFLD is complex and is strongly linked to obesity and type 2 diabetes, with NAFLD being found in up to 70% of diabetic patients[30,31]. NAFLD is also highly associated with cardiovascular and chronic kidney diseases[30,31]. Like CHB immunopathology, NAFLD is an inflammatory disease of the liver, but is caused by an accumulation of fatty acids called steatosis. Given its strong link to obesity, insulin resistance, and heart disease, hepatic steatosis is considered by many to be the manifestation of the "*metabolic syndrome*". This syndrome is comprised of a combination of hypertension, insulin resistance, central or abdominal obesity, dyslipidemia, and low fasting high-density lipoprotein levels in the liver[30].

The hallmark of NAFLD is hepatic steatosis in the absence of excessive alcohol consumption[30]. Unlike HBV infection, hepatic inflammation in NAFLD is sterile and is a result of oxidative stress and the disruption of cellular processes due to lipotoxicity from steatosis[32]. The baseline diagnostic criteria for NAFLD is the presence of steatosis in least 5% of hepatocytes, and is called simple steatosis[30]. The occurrence of only simple steatosis, without hepatic inflammation, is not associated with increased mortality or worsened disease outcomes[30]. However, Non-alcoholic steatohepatitis (NASH) is a more severe stage of NAFLD, where inflammatory damage to the liver results in fibrosis, and can eventually lead to liver cirrhosis and increased mortality[30]. In individuals with NAFLD, an estimated 10-30% will subsequently develop NASH, with approximately 2% of those developing HCC, usually after progression to cirrhosis. However, there are increasing reports of HCC development in non-cirrhotic patients with NASH[30]. Predictions for future NAFLD prevalence in the USA estimate a 21% increase by 2030, with proportions of NASH and HCC cases increasing to 135% and 178%, respectively.[31]

Progression of NAFLD from simple steatosis to NASH is multifactorial. Once triglyceride levels start to overwhelm fat homeostasis, hepatocyte mitochondrial functions begin to generate toxic lipid-metabolites and reactive oxygen species, which both activate inflammatory pathways[33]. Dysregulation of metabolite homeostasis and insulin resistance can also lead to the disruption of several transcription factors, such as sterol regulatory element-binding protein 1 (SREBP1) carbohydrate response binding-element protein (ChREBP), which are also affected by the HBV X protein[34]. Oxidative stress and metabolic dysregulation also affect the endoplasmic reticulum (ER), resulting

in an unfolded protein response (UPR), which can in turn activate SREBP1 pathways[35]. Inflammasome and nuclear factor kinase-B (NF-kB) pathways are activated in NAFLD, leading to TNFα, IL-6, and IL-1β production and chronic inflammatory states[36]. The intestinal microbiome may also play a role in NASH development, as NAFLD patients have been found to have increased intestinal permeability and intestinal overgrowth compared to healthy controls[37]. Bacterial dysbiosis results in increased bacterial metabolite and substrate production, which in turn can disrupt host metabolic homeostasis and inflammatory pathways[37]. Furthermore, NASH phenotypes have been shown to be transmitted between mice through communal microbiome transmission[37]. Insulin resistance is exacerbated in NASH and is associated with several mechanisms present in NAFLD, including disruption of insulin signalling pathways through NF-Kb activation, UPRs, and epigenetic factors[38].

Insulin resistance promotes free fatty acids in the blood, in the form of very-low density lipoprotein cholesterols, and increased gluconeogenesis from adipose tissues[30]. These cholesterols accumulate in hepatocytes and lead to hepatic steatosis. Adipose tissues may also contribute directly to hepatic steatosis through the production of leptin, a proinflammatory hormone that prevents lipid accumulation in non-adipose tissues[39]. Adiponectin is also produced by adipose tissues and blocks inflammatory pathways and improves insulin resistance and liver fibrosis[39]. Obesity is associated with increased leptin, and decreased adiponectin levels[39]. Dietary factors can directly contribute to liver steatosis. Increased caloric, fat, and fructose intake flood the glycolytic pathways and increase substrate concentrations for lipogenesis[40]. Other dietary factors can protect against NAFLD, with Mediterranean type diets leading to reduced hepatic fat content,

even without a reduction in overall body weight[41]. Diet provides metabolites to the intestinal microbiome, which can result in changes in the distribution of particular bacterial species[42]. Intestinal dysbiosis can lead to increased infiltration of the microbial species, which can trigger inflammatory responses[42]. Moreover, genetic predispositions have been implicated in NAFLD development. Several polymorphisms in the patatin-like phospholipase 3 (PNPLA3) gene, which encodes for adiponutrin, have been associated with increased hepatic steatosis, fibrosis, and increased risk of HCC in NAFLD[43]. A loss of function mutation in the human transmembrane 6 superfamily 2 (TM6SF2) gene, which promotes triglyceride removal via low-density lipoprotein secretion, is also associated with hepatic steatosis and increased ALT levels[44]. Overall, NAFLD is a complex multifactorial and multisystem disease, with several factors contributing to its development and progression. Although many patients with HBV infection are at risk of NAFLD and metabolic syndrome, there have been limited studies on the pathophysiology of NAFLD and CHB.

1.5. Summary of clinical assessment and treatment of individuals with NAFLD:

The gold standard for assessing NAFLD progression is a liver biopsy, with steatosis, ballooning hepatocytes, and inflammatory infiltrate, all of which are indicators of NAFLD progression[45]. There are several non-invasive tests that are useful in assessment of disease severity. A liver ultrasound (US) has historically been used to assess for steatosis, although US imaging has low sensitivity in obese patients and in those with minimal steatosis[45]. As noted (see section 1.3), LSM is used to assess for fibrosis, and serum ALT levels are an indicator of hepatocyte death. Additionally, hepatic steatosis

can simultaneously be quantified using transient elastrography adapted with a controlled attenuation parameter (CAP), which uses 50 Hz shear waves to quantify steatosis[27]. Based on prior liver biopsy validation studies, the CAP score for severity of hepatic steatosis are categorized using the following cut offs: (1). S1 (5-33% steatosis) >263; (2). S2 (34-66% steatosis) >28; (3), and S3 (67-100% steatosis) >283 HZ[46]. Liver stiffness measurement to assess fibrosis in patients with NAFLD (without HBV) are as follows: >7.1 kpA (Metavir stage 2), >8.7 (Metavir stage 3), >10,4 kPA (Metavir stage 4 or cirrhosis)[47]. There are no validated studies on the utility of LSM to assess Metavir stage or fibrosis level in patients with both NAFLD and CHB.

In general, improvements to dietary, exercise habits, and weight loss are the first line therapy for NAFLD. There are other treatment options for risk factors associated with NAFLD, especially type 2 diabetes and insulin resistance. A class of anti-diabetic drugs, called thiazolidinediones, improves insulin sensitivity by increasing the transcription of insulin-responsive genes and is recommended for use in NASH[45]. Additionally, bariatric surgery can be considered to reduce weight and visceral adiposity in patients who do not respond to dietary and lifestyle changes, which in turn reduces hepatic steatosis and liver fibrosis[45].

1.6. Interactions between NAFLD and CHB: Several cohort studies have identified a decreased incidence of NAFLD in patients with CHB (Table 2)[43–58]. This relationship is unique to hepatitis B and is only found in patients with CHB infection (based on serum HBsAg positivity), rather than previous exposure (based on presence of anti-HBc and HBsAg negativity or possible OBI)[52]. However, these studies have predominantly taken

place in South-East Asian countries and since HBV genotypes vary by geography, this relationship may be limited to HBV genotypes B and C. Studies using mice genetically modified to express HBV identified decreased serum HBV viral load, HBsAg, and HBeAg, with greater serum and histological signs of liver damage in mice with NAFLD compared to those without NAFLD[63]. However, these studies also used HBV genotype B infection models. Therefore, it is unknown if this negative association between CHB and NAFLD exists across all HBV genotypes.

1.7 Interactions between HBV and metabolic risk factors associated with NAFLD.

The association between hepatic steatosis and HBV is complex and has been evaluated in multiple heterogenous studies (Table 3)[48,51,54,57,62,64–76]. Patients with CHB and NAFLD have increased chances of HCC development, with hazard ratios of 7.3 compared to CHB alone[77,78]. This increased chance of HCC development was consistent regardless of degree of steatosis. A meta analysis of over 4100 patients found a strong negative correlation between hepatic steatosis and HBV DNA levels and qHBsAg[48]. Additionally, HBsAg seroclearance is increased in patients with liver steatosis[55]. An Iranian study found that HBeAg negativity was also greater in patients with concomitant hepatic steatosis, although they did not find any effect on HBV DNA levels[56]. Despite the strong negative association between liver steatosis and HBV viral markers, there are several proposed mechanisms regarding the pathogenesis of HBV-induced hepatic steatosis. Genetic polymorphisms in transmembrane 6 superfamily 2 human gene (TM6SF2), have been associated with increased HBV DNA levels[79]. Zebrafish models expressing HBV X protein had significant increases in sterol

regulatory element-binding protein-1 (SREBP1) and carbohydrate response binding-element protein (ChREBP) gene expression, which regulate cellular cholesterol levels and fat production, resulting in increased fatty acid synthesis[80]. *In vitro* models have also identified upregulation of the fatty acid-binding protein, liver (FABP1) gene, which is linked to the uptake of free fatty acids in hepatocytes, by the HBV X protein[81]. However, HBV has been shown to inhibit apolipoprotein A5 secretion, the knockdown of which decreases intracellular triglyceride levels[82]. No differences in fat accumulation have been identified between HBV genotypes, potentially indicating sequence conservation in HBV X gene regions that leads to hepatic steatosis[48,61]. Although, to date, only genotype B and C have been compared for hepatic steatosis[48,61] . There is evidence for a negative relationship between hepatic steatosis and response to entecavir and pegylated-IFN therapy, although these relationships have been contested by other studies[83–89]. Additionally, there have been very few studies that have observed the effects of different degrees of steatosis on CHB[62]. The mechanisms by which hepatic steatosis decrease serum HBV DNA levels are also unknown.

HBV has also been shown to have a complex relationship with several NAFLD and NASH risk factors. Although the molecular mechanisms between the HBV lifecycle and these NAFLD associated metabolic risk factors have not been identified, conflicting reports have linked CHB to decreased rates of metabolic syndrome and insulin resistance (Table 3). Studies in individuals with the metabolic syndrome have found that CHB patients have lower triglyceride and cholesterol levels compared to hepatitis C patients and healthy individuals[65]. Associations between dyslipidemia and HBsAg seropositivity have also been identified[74]. Decreased HBV DNA levels have been found

in patients with PNPLA3 polymorphisms that are associated with NAFLD[90]. Interestingly, the PNPLA3 I148M polymorphism has also been associated with an increased chance of developing chronic HBV infection, in addition to NAFLD, possibly indicating a role for the PNPLA3 gene in determining host susceptibility to particular low-replicating HBV strains[91]. As previously noted (see section 1.4), obesity is normally associated with decreased adiponectin levels. However, CHB patients have both increased adiponectin levels and rates of obesity when compared to non-CHB controls[70]. This study also found adiponectin to be positively associated with HBV DNA levels, indicating that, even in obese patients, HBV may be able to upregulate adiponectin for its anti-inflammatory effects.

Innate immune responses in NAFLD may be the primary mechanism by which HBV is affected by NAFLD. A study using mice expressing HBV found that TNFα, IFN-β, and IL-6 levels and TLR4 expression were all increased in NAFLD[92]. All of these cytokines are associated with antiviral immune responses, either through the activation of T-cell responses or by initiating signalling pathways that lead to antiviral responses or cell death[93–95]. This study indicates that the innate immune response from NAFLD is a mechanism for the inhibition of HBV[92], although not observed in human studies. Additionally, the host microbiota may be a major cause for the impact of NAFLD on HBV[37]. As previously mentioned, host immune control of HBV is also linked to gut microbiota composition. Therefore, it is possible that the dysbiosis and intestinal permeability seen in NAFLD may be a key factor in supressing viral replication. NAFLD has also been associated with decreased rates of HBV immunity following vaccination in pediatric patients[96,97]. Together, these studies suggest that NAFLD, and associated

intestinal dysbiosis, may suppress the adaptive immune response while also boosting innate immune responses to HBV.

1.8 Rationale, Hypothesis and Aims: Most studies on the relationship between NAFLD and CHB have focused on a single factor relating to the pathogenesis of NAFLD or considered only the presence or absence of NAFLD. A study by Hui *et al.* found that in patients with NAFLD and CHB the severity of hepatic steatosis and the presence of insulin resistance were associated with a significant decrease in viral load[62]. However, the effects of other NAFLD associated metabolic risk factors (i.e., obesity, visceral adiposity, and metabolic syndrome) on CHB viral factors have not been studied in patients with NAFLD. Furthermore, genetic characteristics of the HBV, such as genotype, genetic diversity, or the presence of mutations, as well as novel biomarkers of viral replication, such as viral pgRNA and mRNA, have not been studied in NAFLD. Lastly, as NAFLD and CHB are complex inflammatory diseases affecting the liver, a better understanding of the inflammatory state seen in patients with both NAFLD and CHB is necessary to understand the progression of these diseases and their impact on one another.

The objective of this thesis is to determine the effects of metabolic factors associated with NAFLD on HBV genetic characteristics, replication markers, host antiviral specific immune responses, and host immune cytokine. We hypothesize that individual NAFLD associated metabolic risk factors will be associated with differences in HBV replication markers, viral genetic characteristics, HBV specific immune responses, and host cytokine patterns in patients with CHB and NAFLD.

Table 1: Standard and novel liver clinical tests and viral biomarkers used to monitor chronic hepatitis B and non-alcoholic fatty liver disease progression.

Disease	Clinical marker	Use
CHB	qHBsAg	A marker of transcription activity of cccDNA and genome integrated HBV DNA and indicator of patient response to anti-viral treatments.[4,13]
	HBeAg	An immunomodulatory viral protein and marker of HBV activity and viral replication.[13]
	Anti-HBe	Indicative of HBeAg negativity and seroconversion.[4,9,13]
	HBV DNA	High viral DNA is associated with progressive liver disease.[4,13]
	HBV RNA	Viral RNA indicates transcription activity of cccDNA and genome integrated HBV, can be used to identify treatment endpoints.
	Anti-HBs	Indicative of immunity to HBV.[4,13]
	Anti-HBc	Anti-HBc IgM is indicative of a recent infection, anti-HBc IgG indicates the infection is chronic and ongoing [9,13,14]
	Genotype	Can be indicative of response to treatments and progression of liver disease.[10]
NAFLD	Controlled attenuation parameter (CAP)	Provides a non-invasive measure of liver steatosis.[27]
	Liver stiffness measurement (LSM)	Provides a non-invasive measure of liver fibrosis.[27]
	Liver biopsy*	Invasive, marker of histological inflammation and fibrosis in CHB and steatosis in NAFLD
	serum alanine aminotransferase (ALT)	Increased levels are indicative of active liver damage.[29]

*Liver biopsy is used in both CHB and NAFLD.
CHB – Chronic hepatitis B; NAFLD – non-alcoholic fatty liver disease; qHBsAg – quantitative hepatitis B surface antigen; HBeAg – hepatitis B virus E antigen; HBV – hepatitis B virus; Anti-HBs – hepatitis B surface antibody; Anti-HBc – hepatitis B core antbody; IgM – immunoglobulin M; IgG – Immunoglobulin G; CAP – controlled attenuation parameter; LSM – liver stiffness measurement; ALT – alanine aminotransferase. Adapted from Coffin *et al*. 2019[13].

Table 2: Previous studies on the relationship between NAFLD, hepatic steatosis and HBV biomarkers

Study	n with CHB	Study type	NAFLD associated variable	HBV factor	Relation-ship
Machado *et al.* 2012[48]	4100	Meta-analysis	Liver steatosis	HBeAg	None
				Genotype B or C	None
				HBV DNA	Negative
Wang et al. 2014[49]	3212	Retrospective cohort	Liver steatosis	HBeAg	Negative
				HBV DNA	Negative
				Intrahepatic HBsAg	Negative
Chu *et al.*2013[55]	155	Prospective cohort	Liver steatosis	HBsAg clearance	Positive
				Anti-HBsAg seroconversion	None
				HBV DNA post HBsAg clearance	None
Poortahmasebi *et al.* 2014[56]	160	Cross-sectional	Liver steatosis	HBeAg	Negative[1]
				HBV DNA	None
Wang et al. 2008[57]	50	Cross-sectional	Liver steatosis	HBsAg	None
Zheng *et al.* 2010[58]	204	Prospective cohort	Liver steatosis	HBV DNA	Negative
Cai *et al.* 2018[59]	1236	Cross-sectional	Liver steatosis	HBV DNA	None
				HBeAg	None
Shi *et al.* 2008[60]	1915	Retrospective cohort	Liver steatosis	HBV DNA	None
				HBeAg	None
Lesmana *et al.* 2012[61]	174	Cross-sectional	Liver steatosis	HBV DNA	None
				HBeAg	None
				Genotype B or C	None
Hui *et al.* 2018[62]	1202	Cross-sectional	Steatosis severity	HBV DNA	Negative
Xiong *et al.* 2017[50]	8272	Meta-analysis	NAFLD incidence	CHB diagnosis	Negative
Joo *et al.* 2017[51]	3,926	Prospective cohort	NAFLD incidence	CHB diagnosis	Negative
Zhong *et al.* 2018[52]	631	Retrospective cohort	NAFLD incidence	CHB diagnosis	Negative
Wang *et al.* 2019[53]	152	Prospective cohort	NAFLD incidence	HBsAg	None
Wong *et al.* 2012[98]	91	Cross-sectional	NAFLD incidence	CHB diagnosis	Negative
				HBeAg	None
				HBV DNA	None
				Genotype B or C	None

CHB – Chronic hepatitis B; NAFLD – non-alcoholic fatty liver disease; HBV – hepatitis B virus; qHBsAg – quantitative hepatitis B surface antigen. 1. Age matched samples showed no association between steatosis and HBeAg status.

Table 3: Previous studies on the relationship between metabolic syndrome, and /or components of metabolic syndrome, and HBV infection or viral load.

Study	n with CHB	Study type	NAFLD associated variable	HBV factor	Relationship
Hui *et al.* 2018[62]	1202	Cross-sectional	Metabolic syndrome	HBV DNA	None
Wong *et al.* 2012[54]	91	Cross-sectional	Metabolic syndrome	CHB diagnosis	Negative[1]
Jan *et al.* 2006[71]	5995	Cross-sectional	Metabolic syndrome	HBV infection	Negative
Luo *et al.* 2007[72]	858	Cross-sectional	Metabolic syndrome	HBV infection	Negative
Li *et al.* 2013[73]	3408	Case series	Metabolic syndrome	HBV infection	None
Chung *et al.* 2014[74]	521	Cross-sectional	Metabolic syndrome	HBV infection	Negative[2]
Jinjuvadia *et al.* 2014[75]	593594	Cross-sectional	Metabolic syndrome	HBV infection	Negative
Zhou *et al.* 2014[76]	480	Retrospective cohort	Metabolic syndrome	HBV infection	Positive
Jarcuska *et al. 2014*[64]	66	Cross-sectional	Metabolic syndrome	HBV infection HBV DNA	None Positive
Janicko *et al.* 2014[65]	55	Cross-sectional	Metabolic syndrome	HBV infection	None
Choi *et al. 2015*[66]	209	Retrospective cohort	Metabolic syndrome	HBV infection	Negative[2]
Liu *et al.* 2012[67]	1123	Prospective cohort	Dyslipidemia	HBV infection	Negative
Chen *et al.* 2010[68]	6133	Retrospective cohort	Dyslipidemia	HBV infection	Negative
Wang et al. 2008[57]	50	Cross-sectional	Insulin resistance	HBV infection	None
Lee *et al.* 2012[69]	603	Retrospective cohort	Insulin resistance	CHB diagnosis	Positive
Chiang *et al.* 2013[70]	3587	Prospective cohort	Obesity Dyslipidemia	HBV DNA HBV DNA	Negative[3] Negative[3]
Joo *et al.* 2017[51]	3,926	Prospective cohort	Cholesterol	HBsAg	Negative

CHB – Chronic hepatitis B; NAFLD – non-alcoholic fatty liver disease; HBV – hepatitis B virus; qHBsAg – quantitative hepatitis B surface antigen 1. Age matched samples showed no association between steatosis and HBeAg status. 2. Correlation found only in male patients. 3. In HBeAg positive patients only.

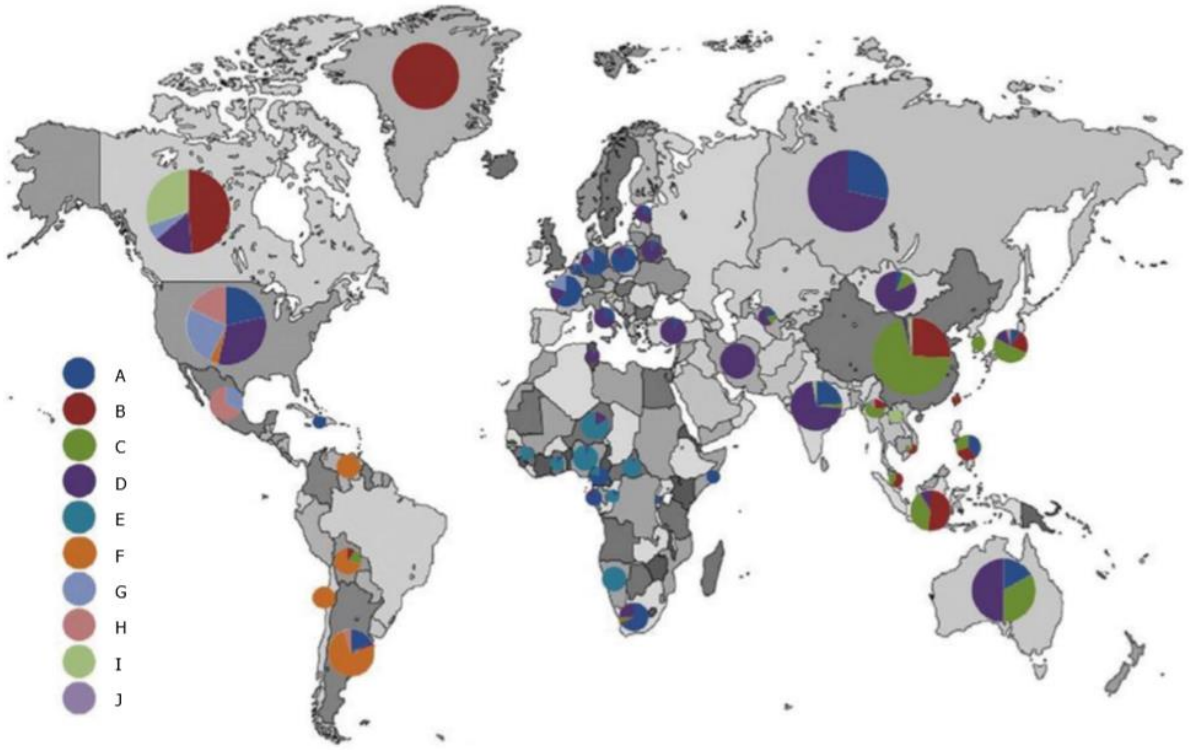

Figure 1: Geographical distribution of hepatitis B virus genotypes. Source: Sunbul 2014[10].

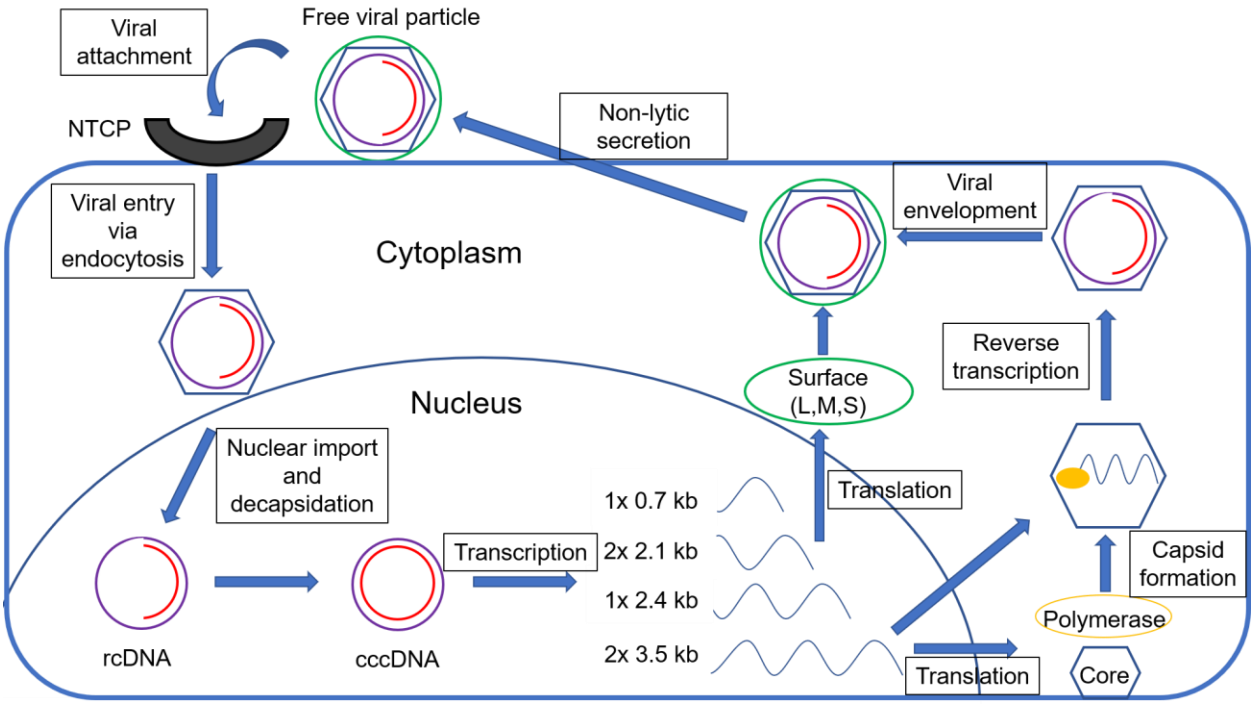

Figure 2: An illustration of the hepatitis B virus lifecycle. The virus is internalized by the hepatocyte through the interaction of the HBV envelope and sodium taurocholate cotransporting polypeptide (NTCP). The viral capsid is transported to the nucleus, where it deposits the partially double-stranded viral genome, relaxed circular (rc)DNA. Host genomic histones interact with rcDNA to create a stable, fully double stranded replication template called covalently closed circular (ccc)DNA. The cccDNA produces several viral RNA transcripts, with a 3.5 kb RNA product called pregenomic (pg)RNA. The HBV core and polymerase proteins are encoded by pgRNA. The P binds to pgRNA, signaling the capsid to assemble from core proteins, then reverse transcribes the pgRNA into rcDNA. The envelope is made up of the small, medium, and large surface proteins, which are produced from the 2.1 and 2.4 kb viral mRNA. The envelope forms around the viral capsid and the new viral particle leaves the cell via secretory systems or viral capsid is recycled to the nucleus to replenish the HBV cccDNA pool.

Chapter 2:

Characterization of Hepatitis B Virus and Host Cytokine Patterns in a Multiethnic Cohort of Patients with Non-Alcoholic Fatty Liver Disease and Chronic Hepatitis B

2.1 Abstract: Background & Aims: Individuals with chronic hepatitis B (CHB) and non-alcoholic fatty liver disease (NAFLD) are at higher risk of liver fibrosis but the pathogenesis is unclear. Methods: A multiethnic cohort of 48 patients with comorbid CHB and NAFLD were prospectively recruited from 3 liver/infectious disease clinics. Standard clinical tests included non-invasive test for fibrosis/steatosis, HBV DNA and RNA were quantified from plasma via qPCR. Viral genotypes and mutations were analyzed via direct population and next-generation sequencing analysis. Cytokine levels were measured in serum and in supernatant of PBMCs following *ex-vivo* stimulation with viral antigens, with a 13-plex immunoassay (Luminex). Appropriate statistical analyses were done using R commander. Results: In 48 subjects (64.5% Male, 60.4% South East Asian, mean age of 44.4±10.3. 12.5% of patients were infected by HBV genotype A, 18.8% by B, 45.8% by C, 16.7% by D, and 6.3% by E. The majority of patients were HBeAg negative (93.8%) with mean HBV DNA log 3.2±1.7 IU/mL, high liver steatosis (mean CAP 302.3±51.9 dB/m), and minimal fibrosis (mean LSM 5.8±2.0 kPa). Levels of qHBsag was significantly higher in obese patients (p=0.012). Mutations at residues associated with immune escape, anti-viral drug resistance, HBeAg negativity, and liver disease progression were identified in several. HBV C gene mutations, F24Y and E64D, were increased, while frequency of I140M surface (S) gene immune escape mutation was decreased in patients with NAFLD associated metabolic risk factors. Additionally, S gene diversity was increased in subjects with metabolic syndrome and/or diabetes (p=0.008). Patients with metabolic syndrome had increased serum IFN-γ (p=0.027) and IL-8 (p=0.012) levels. Th1/Th2 responses to HBV antigen stimulation were not different between NAFLD patients with or without associated

metabolic factors, and there was no difference compared to unstimulated controls. Conclusion: In this multiethnic cohort study of patients with comorbid HBV and NAFLD, individuals with the metabolic syndrome exhibit unique viral genetic characteristics and lower HBV specific immune responses. The different inflammatory state associated with the metabolic syndrome may be a mechanism responsible for the higher risk of liver disease progression seen in patients with both NAFLD and CHB.

2.2 Background: CHB and NAFLD are high prevalence inflammatory liver diseases. There are estimated to be 250 million HBsAg positive CHB carriers globally, which are linked to nearly 800,000 cases of HCC[1]. Given the increase in obesity rates, to which NAFLD is closely linked, NAFLD has become the most common liver disease among the general population[30]. While liver inflammation in CHB is due to complex and dynamic interactions between the HBV and the host immune system, the inflammatory response in NAFLD is caused by cellular steatosis that leads to disruption of metabolite homeostasis, generation of free radicals, and changes to cell regulatory systems[33,35,36].

Several studies have identified links between CHB and NAFLD, as well as metabolic factors associated with NAFLD (i.e., diabetes, dyslipidemia, central obesity, hypertension, collectively referred to as the metabolic syndrome). Multiple epidemiological studies have found that patients with CHB have a lower incidence of NAFLD[50–52,98]. However, patients with evidence of prior HBV infection and natural immunity (i.e., HBsAg negative with anti-HBs and/or core anti-HBc) show no differences to otherwise healthy controls, indicating a protective mechanism of HBV infection against NAFLD[52]. The presence of hepatic steatosis has been linked to decreased

levels of HBV DNA and HBsAg, decreased HBeAg positive rates, and increased rates of HBsAg/anti-HBs seroconversion[48,49,55,56,62]. However, these results have predominantly been demonstrated in Asian males and several studies contest the relationship[33,56–68,82]. High viral load (HBV DNA levels), long duration of infection, and presence of cirrhosis, have been shown to independently increase the chance of diabetes development, while another study indicated that CHB may have a protective effect against the metabolic syndrome[101,102]. Patients with NAFLD have also been found to have decreased responses to the approved recombinant HBsAg vaccine for HBV, suggesting that NAFLD can affect HBV-specific immune responses[96,97]. However, the mechanisms responsible for these interactions are unknown.

CHB patients with NAFLD are at increased risk of HCC development[78]. Various epidemiological studies have reported conflicting results regarding differences in HBV antiviral treatment associated with NAFLD[83–88]. Further research is needed to understand pathogenesis and inform clinical management guidelines for individuals with CHB and NAFLD. This study aims to determine the impact of NAFLD and associated host metabolic factors on HBV viral characteristics (i.e., HBV quasispecies, variants and viral replication markers) and host immune patterns. We hypothesize that in patients with CHB and NAFLD, the severity of hepatic steatosis, and presence of metabolic syndrome (i.e., diabetes, dyslipidemia, central obesity), will impact viral characteristics and host antiviral immune responses.

2.3. Methods

2.3.1 Patient recruitment and clinical evaluation: Patients were prospectively enrolled from 3 tertiary referral liver clinics. All data received were collected under a University of Calgary Conjoint Ethics Research Board (CHREB) approved protocol (Ethics ID# REB16-0041). All samples were collected and sample/data analysis was performed under CHREB Ethics ID# REB16-1862. Eligible participants provided informed consent and the study was approved at each participating site's local research ethics board. Inclusion criteria included between 18-60 years of age, confirmed diagnosis of NAFLD or NASH based on clinical consensus guidelines[103], confirmed diagnosis of CHB (HBsAg positive for >6 months), no cirrhosis, no prior or active anti-HBV treatment, and absence of liver disease from other sources, including excessive alcohol intake[45]. Baseline demographic and relevant data for NAFLD and CHB diagnoses were collected from the patient clinical history and included body mass index (BMI), waist circumference, and controlled attenuation parameter (CAP) with liver stiffness measurement (LSM) by transient elastography (Fibroscan®, Echosens, Paris, France). Standard clinical tests included serum alanine transaminase (ALT) levels, qHBsAg levels (qHBsAg, Abbott Diagnostics, Abbott Park, Illinois, USA, lower limit of detection (LLOD) 0.05 IU/mL), HBV DNA (Abbott Molecular m2000 RealTIMe System, Abbott Park, Illinois, USA, LLOD 10 IU/mL), HBeAg and HBeAb status (Abbott Diagnostics, Abbott Park, Illinois, USA), serum Mg, fasting glucose, ferratin, and vitamin D levels. Patients were diagnosed with metabolic syndrome based on at least 2 of the following: (1). Essential Hypertension; (2). Insulin resistance or Type 2 Diabetes; (3). Central (abdominal) obesity; (4). Dyslipidemia (i.e., high hypertriglyceridemia or low-

density lipoprotein levels)[103,104]. Blood samples were collected from patients at baseline and during a 6-12 month follow-up visit. Approximately 40 mL of whole blood was collected using EDTA vacutainers® (Becton Dickinson Diagnostics, East Rutherford, New Jersey, USA), with an additional 10 mL collected for serum. Samples were either processed within 2 hours, if collected locally, or shipped to the central processing lab. Plasma and peripheral blood mononuclear cells (PBMCs) were isolated from whole-blood and stored in -80^{o}C or liquid nitrogen, respectively.

2.3.2. Nucleic acids isolation, sequencing, and quantification: Total DNA was extracted from 500µL of plasma using a standard phenol-chloroform extraction protocol and resuspended in 30µL of RNA/DNA free water (Appendix C). All DNA extractions were performed in parallel with negative controls. HBV DNA was amplified via direct PCR using TaqMan^{tm} polymerase (purchased from New England Biolabs, Ipswich, Massachusetts, USA) containing 5µL of extracted DNA template, primers specific to the preC/C, and preS/S regions of the HBV genome (Table S1-S2), with premixed dNTPs (purchased from New England Biolabs, Ipswich, Massachusetts, USA). A follow-up nested TaqMan^{tm} PCR amplification was performed using 2µL of the direct round PCR product. Following amplification, all PCR products were analyzed on a 1% agarose gel with Sybr Safe^{tm} (purchased from Life Technologies, Carlsbad, California, USA). HBV DNA positive samples were gel purified using a QIAquick™ gel extraction kit (purchased from Qiagen, Venlo, Limburg, Netherlands), according to manufacturer protocols, and submitted for Sanger sequencing (University of Calgary Sequencing Services, Calgary AB, Canada). The HBV genotype was determined using BLAST and

HBV genotyping tool (National Center for Biotechnology Information, Bethesda, Maryland, USA). Clonal sequencing was performed using previous preS/S and preC/C gel extractions as per a previously published protocol (Appendix C)[105]. For next-generation sequencing (NGS), NGS adapter primers, which covered the S and C regions (Table S1), were used in a Phusion (purchased from New England BioLabs, Ipswich, Massachusetts, USA) polymerase-based PCR, with dNTPs (purchased from New England Biolabs, Ipswich, Massachusetts, USA), to amplify gel-extracted preS/S and preC/C. The NGS amplicons were gel purified using a QIAquick™ gel extraction kit (purchased from Qiagen, Venlo, Limburg, Netherlands), according to manufacturer protocols, and submitted for sequencing via the MiSeq (purchased from Illumina, San Diego, California, USA) platform. A single PCR amplified preS/S and preC/C HBV clone was used as an internal control to determine the NGS error rate (mean error rate calculated as <1%)[106]. All conventional PCRs used DNA extracted from known high viral load patient plasma as a positive control. All sequences were aligned by the ClustalΩ method (gap opening penalty = 15, gap extension penalty = 6.66) in MEGA version 7. Genetic diversity between individual viruses in the preC/C and preS/S regions in each patient was calculated via the compute mean diversity function in MEGA 7 (1000 bootstrap replicates). A Kimura 2-parameter model was selected to allow for differential rates of transitions and transversions between viral variants without presumption of the base proportions of nucleotides. To assess HBV DNA (viral load), HBV surface gene DNA was amplified in triplicate from 5µL of extracted DNA template and quantified using perfeCTa Fastmix II (purchased from Quanta Biosciences Inc, Gaithersburg, Maryland, USA, LLOD 100 copies/mL) qPCR. Plasmid standards with concentrations from 10^7-10^0

copies/μL were used as positive controls and to generate a standard curve for HBV S gene DNA quantification.

Total RNA was extracted from 500μL of plasma via a standard Trizol-chloroform extraction protocol and resuspended in 30μL of RNAse/DNAse free water (Appendix C). All RNA templates were digested with RNase-free DNase (purchased from Qiagen, Venlo, Limburg, Netherlands), according to manufacturer protocols. Rapid amplification of complimentary ends (RACE) cDNA conversion was performed on total HBV mRNA using a Superscript II™ kit (purchased from Life Technologies, Carlsbad, California, USA), according to manufacturer protocols, and quantified by perfeCTa Fastmix II (purchased from Quanta Biosciences Inc, Gaithersburg, Maryland, USA) qPCR[107,108]. HBV pgRNA was reverse transcribed using a total-RNA conversion qScript™ cDNA synthesis kit (purchased from Quanta Biosciences Inc, Gaithersburg, Maryland), according to manufacturer protocols, and was quantified using qPCR. All RNA extractions and cDNA conversions were performed alongside negative mock controls. Plasmid standards with concentrations from 10^7-10^0 copies/μL were used as positive controls and to generate a standard curve for both pgRNA cDNA and RACE cDNA quantification (lower limit of detection of 450 copies/mL).

2.3.3. Immunological assays: Total PBMCs were thawed in a 37^{o}C water bath and reconstituted in culture wells with 1 mL RPMI 1640 medium (purchased from ThermoFisher Scientific, Waltham, Massachusetts, USA) with 10% Fetal Bovine Serum (FBS, purchased from ThermoFisher Scientific, Waltham, Massachusetts, USA) per 1,000,000 cells for 24 hours. PBMCs were either unstimulated or stimulated in triplicate

with 5 ug/mL HBcAg (purchased from American Research Products Inc., Boston, Massachusetts, USA), HBsAg (purchased from American Research Products Inc., Boston, Massachusetts, USA), or phytohaemagglutinin (purchased from PHA, Merck & Co., Kenilworth, New Jersey, USA) as a positive control. After 72 hours, PBMC supernatants were collected and pooled for analysis. Pooled supernatant from PBMCs, patient serum samples, and healthy control serum samples were measured in duplicate using a commercial high sensitivity T-cell discovery multiplex cytokine assay that measured GM-CSF, IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, MCP-1 and TNFα (Eve Technologies, Calgary, Alberta, Canada).

2.3.4 Statistical analysis: Patients were stratified based on their demographic, clinical, and HBV characteristics. Age and sex matched patients were stratified based on their CAP score and presence of metabolic syndrome for immunological analysis. Categorical cut-offs for CAP score were <280 dB/m for low steatosis and >280 dB/m for high hepatic steatosis, and LSM >7.3 kPA, which is indicative of moderate liver fibrosis without cirrhosis[109]. The subjects BMI and waist circumference were used to determine obesity class and central obesity status, based on ethnicity specific cut-offs (Table S3)[110,111]. As only 3 HBeAg positive patients were recruited, statistical analysis was only performed on HBeAg negative patients. Variables were analyzed using the Kruskal-Wallis test, multiple linear regression, and Chi-square or Fischer's exact test. All statistical tests were performed using R commander.

2.4 Results

2.4.1 Summary of demographic and clinical characteristics in CHB patients with NAFLD:

In total, 48 untreated patients were prospectively recruited into the study (including 10 with follow-up/serial samples collected at 6-12 months intervals). The majority were male (64.6%), mean age of 44.5±10.4, 60.4% Asian ethnicity (Table 4). For HBV genotype, 12.5% of patients were infected by A, 18.8% by B, 45.8% by C, 16.7% by D, and 6.3% by E (Table 5). The mean CAP score for entire cohort was 303.1±53.1 dB/m, and the majority of patients had CAP scores >280 (59.6%), indicating high liver steatosis. However, mean ALT (42.1±25.4 IU/mL) and LSM scores (5.9±1.9 kPA) showed that despite high hepatic steatosis and active liver damage, most patients had minimal liver fibrosis as determined by LSM. There were no differences in viral characteristics or immune factors across subject ethnic backgrounds, but after adjusting for ethnic-specific BMI cut-offs, patients of South-East Asian decent showed a significantly lower rates of obesity (p=0.029). Based on ethnicity specific obesity cut-offs, 43.8% of patients were classified as obese based on BMI.

2.4.2. Analysis of HBV replication markers: The majority of subjects were HBeAg negative (93.8%) with mean HBV DNA 3.4±1.3 log10 copies/mL (range of 0–6.3 log10), and most had undetectable pgRNA (88.9%, mean 0.4±1.0 log10 copies/mL) and mRNA (66.7%, mean1.0±1.7 log10 copies/mL) levels. In 3 HBeAg positive patients the mean HBV DNA level was 8.8±0.3 log10 copies/mL (range of 8.4–9.2 log), with HBV mRNA and pRNA detected in 2/3 patients (1/3 the sample was shipped from a collaborating lab and inadvertently frozen which likely affected viral RNA detection). Neither HBeAg

positive or negative subjects showed significant differences in the presence or absence of metabolic factors associated with NAFLD (Table 4). The degree of liver steatosis as assessed using CAP, the presence of metabolic syndrome, or its components, did not significantly relate to HBV viral factors assessed (Table 6). However, obese patients (based on ethnicity specific BMI cut-offs), showed significantly higher median qHBsAg levels (median rank=19.38) compared to non-obese patients (median rank=11.44, p=0.012). Regression analysis indicated that patient BMI (i.e., higher obesity status) was a significant predictor of higher qHBsAg levels (p=0.008).

2.4.3 Analysis of HBV genotype and quasispecies by direct population and next generation sequencing in 45 **HBeAg negative CHB patients with NAFLD:** The HBV preS/S and preC/C genes were PCR-amplifed and sequenced in 93% (42/45) and 77% (35/45) of patients, respectively, with at least one gene being amplified in each patient sample collected. All HBV genotypes A to E were detected, with C as the most prevalent (43.8%). There were no significant differences in obesity, metabolic syndrome, or CAP score between different HBV genotype infections (Table 5).

In 8/35 (22%) subjects, clinically relevant mutations in the HBV preC region associated with HBeAg negativity (i.e., G1896A, G1899A) were identified and 18/35 patients had viral variants with C gene mutations associated with HCC development (i.e., F24Y, E64D, E77Q, A80I/V/L, L116I) with a median of 4.4% (range of 1-99%, Figure 3A-C). Clinically relevant mutations in the HBV preS/S region associated with entecavir, telbivudine, or lamivudine resistance (i.e., F161H/L, L173F, L175F, L176V, S193F/L) were found in 25/42 subjects analyzed (median 5.4%, range of 1-99%) of viral

quasispecies with these mutations. Immune escape mutations (i.e., P127L/T, M133L/I/T, T140I, S143L, D144A/E, G145R, E164D/G, P178R) were also found in 33/42 subjects (median 6.9%, range of 1-99%, Figure 3A-C). The vaccine escape G145R preS/S mutation was also identified in 4/42 patients albeit in very low proportions (median 0.019%, range 0.011-0.029%). In subjects with metabolic syndrome or obesity a higher median rank proportion of clones with the F24Y (p=0.02) and E64D mutations (p=0.007), respectively, was observed (Figure 3B-C). Patients with CAP >280 (i.e., high steatosis) also had lower proportions of the preS/S T140I mutation (Figure 3A).

The HBV S gene viral diversity ranged from 0.023-0.118 (median=0.046) and 0.031-0.106 (median=0.041) in the HBV C gene. HBV S gene diversity, determined by NGS, was significantly higher in diabetic patients (p=0.008, Table 6), but diabetes was not found to be a true predictor of S gene diversity by regression analysis (p=0.102, Table 7). Viral diversity in either C or S gene, by any method, was not significantly different between subjects with or without other metabolic syndrome factors associated with NAFLD.

2.4.4. Summary of serum cytokine and anti-HBV specific immune responses: In total, serum from 20 HBeAg negative, treatment naïve patients were analyzed using a multiplex discovery assay for panel of serum cytokines/chemokines. Patients with diabetes had significantly higher IL-8 levels than patients without diabetes (p=0.008, Table 6), and diabetes was found to be a significant predictor for serum IL-8 according to multiple linear regression analysis (p=0.002, Table 7). Likewise, the presence of

metabolic syndrome was associated with increased IL-8 (p=0.012) as well as IFN-γ (p=0.027) levels (Table 6). The presence of the metabolic syndrome was found to be a significant predictor for increased IL-8 (p=0.014), but not for IFN-γ levels (p=0.973, Table 7). The levels of IL-8 or IFN-γ were not significantly associated with any other specific component of the metabolic syndrome. Obesity status and the severity of hepatic steatosis, as determined by CAP measurement, were not associated with any differences in inflammatory cytokine patterns.

HBV specific responses were compared in age and sex matched subjects with or without severe steatosis and/or metabolic syndrome. In total, 8 CHB/NAFLD patients (4 with CAP >280 and metabolic syndrome and/or obesity) were selected. Compared to unstimulated (HBV negative) controls, PBMCs from subjects stimulated by HBsAg and HBcAg did not have significantly higher IFN-γ or IL-4 levels (Figure 4). However, non-specific stimulation with PHA did show higher IFN-γ and IL-4 levels compared to unstimulated controls. When comparing the fold change from unstimulated to stimulated PBMCs, no differences were found in patients with CAP >280 (n = 4) vs. <280 (n = 4), patients with metabolic syndrome (n = 4) vs. without metabolic syndrome (n = 4), or obese (n = 2) vs non-obese (n = 6) patients for either IFN-γ or IL-4 (Figure 5A-C).

2.5. Discussion

This prospective study of multi-ethnic cohort of CHB and NAFLD patients aimed to characterize the relationships between hepatic steatosis and metabolic syndrome factors associated with NAFLD and HBV viral factors (i.e., viral replication markers, genotype, mutations, diversity), host inflammatory cytokine patterns, and HBV-specific

T-cell responses. We found that the majority of CHB patients with NAFLD recruited were HBeAg negative with low-level HBV DNA. Most of the assessed markers of HBV replication were not different between patients with NAFLD associated metabolic risk factors, except for HBsAg levels which were higher in obese patients. Some viral mutations appeared in different frequencies in patients with NAFLD associated metabolic disorders, and S gene diversity was increased in metabolic syndrome patients, possibly indicating altered immune selection. The presence of metabolic syndrome and diabetes were also associated with increased serum IFN-γ and IL-8. However, metabolic factors did not appear to affect HBV specific antiviral immune responses.

This data is consistent with other large epidemiological studies, which have reported an inverse relationship with hepatic steatosis and HBV replication and HBeAg positivity[48,49,55–59,61,62]. Interestingly, despite low level HBV DNA, patients classified as obese based on BMI had increased qHBsAg levels. Previous studies have found that patients with low viremia, but high qHBsAg had increased rates of cirrhosis and HCC[112]. If obesity and higher qHBsAg levels are positively related in HBeAg negative patients, despite low-level HBV DNA, then it could represent either increased selection for low-replicating viral quasispecies or the presence of host integrated HBV. Host integrated HBV is not capable of replication due to a separation of the C gene from its promoter, but can still transcribe HBsAg, and is associated with HCC development[21,113]. An increased number of integrated HBV genomes in obese patients may be a potential factor underlying the increased risk of HCC and liver fibrosis progression in patients with NAFLD and CHB.

Our cohort included all HBV major HBV genotypes (A-E). However, the majority of viral genotypes identified were B and C, which was consistent with the ethnic composition of patients in this study (i.e., predominantly South-East Asian where genotypes B and C are endemic)[10]. The specific viral genotype did not relate to metabolic factors associated with NAFLD, indicating that the mechanisms for interaction between CHB and NAFLD are not genotype specific. Some genotypes were underrepresented in our cohort, warranting continued study on the effects of HBV genotype in NAFLD.

Differences in clinically relevant mutations in both the preS/S and preC/C regions of the HBV genome were identified in NAFLD and CHB patients with associated metabolic risk factors. Previous studies have found decreased efficacy of anti-HBV therapy in patients with CHB and NAFLD[84,87,88]. While viral mutations at sites associated with antiviral drug-resistances were identified in several patients, these mutations were mostly found in very low proportions in most patients and likely would not confer resistance or explain differences in treatment response. Likewise, HBeAg positivity has been negatively associated with NAFLD, but a minority of patients in this study had mutations associated with HBeAg negative HBV strains. This indicates that HBeAg negativity in NAFLD is likely due to innate immune-control mechanisms, rather than immune selective pressure/HBeAg escape variant.

In several patients HBV variants were detected with mutations at sites associated with increased risk of HCC development or immune escape phenotypes. The F24Y and E64D mutations were detected more frequently in patients with metabolic syndrome and obesity, while the frequency of the T140I mutation was decreased in patients with high

steatosis. The F24Y and E64D mutations affect CD8 T-cell epitopes of HBcAg, and are thought to be associated with immune escape, and are also associated with increased liver disease progression, while T140I is associated with immune escape phenotypes[114]. The differences in the proportions of these mutations in NAFLD patients may indicate that the presence of metabolic factors associated with NAFLD trigger mechanisms for immune selection on HBV in NAFLD patients. Given that the proportion of HBV variants with C gene mutations were higher, and S gene mutations was lower, in patients with NAFLD and metabolic syndrome, these bystander inflammatory mechanisms could create HBV C gene selective pressures. Additionally, viral diversity in the HBV S gene was increased in diabetic patients, possibly indicating altered selective pressure on HBsAg by the host immune system.

Patients with metabolic syndrome and diabetes had significantly higher levels of serum IL-8 and IFN-γ. Serum IFN-γ levels have been shown to increase with central obesity, liver fibrosis and HCC[115]. However, serum IFN-γ levels did not correlate with liver fibrosis, ALT, HBV viral load, or qHBsAg in this study, possibly indicating an alternate mechanism of action for IFN-γ in CHB and NAFLD patients with metabolic syndrome. IL-8 is upregulated in NASH phenotypes and is secreted by macrophages to recruit and activate neutrophils to sites of inflammation[115]. However, IL-8 is also upregulated by HBV and is known to desensitize HBV to IFNs[116]. A study by Gong *et al.* also found that CHB patients with hepatic steatosis and insulin resistance had increased risk of pegylated-IFN treatment failure compared to those without steatosis and insulin resistance[88]. Therefore, the upregulation of IL-8 in patients with CHB and NAFLD, as

seen in this study, could explain the decrease in HBV sensitivity to pegylated-IFN that have been previously observed.

The increase in IL-8 with metabolic syndrome may also explain the increase in S gene diversity in diabetic patients (Figure 6). It is known that IFN stimulates the production of the APOBEC3 family of proteins via the STAT3 pathway[117]. APOBEC3 proteins limit viral replication by inducing hypermutations that generate non-viable progeny[117,118]. The HBsAg has been demonstrated to inhibit the anti-viral mechanism of APOBEC3G by interacting with the STAT3 pathway[119]. The mechanism for this interaction is unknown, although it is possible that IFN creates a selective pressure for particular HBsAg phenotypes that inhibit APOBEC3G via STAT3. IL-8 is also known to prevent the inhibition of HBV by IFN, and has been shown to interact with the STAT3 pathway in some types of cancer[116,120]. If the IFN disrupting mechanism of IL-8 and HBsAg is similar, then the increase of IL-8 could alleviate the selective pressure of IFN for particular HBsAg phenotypes, thereby allowing the virus to generate viral variants with more diverse HBsAg phenotypes.

PBMCs isolated from CHB and NAFLD patients stimulated *ex vivo* with HBsAg and HBcAg did not show significantly different Th1 and Th2 immune responses, based on presence or absence of metabolic risk factors associated. Our finding is consistent with a previous study that identified no relationship between previous HBV exposure, with subsequent immunity, and NAFLD[52]. However, PBMC responses to HBcAg and HBsAg were also no different compared to unstimulated controls, while PHA stimulated PBMCs had strong Th1 and Th2 responses. This lack of a response to HBV, despite strong response to a non-specific mitogen, may reflect alternative or exacerbated

mechanisms for HBV specific T-cell immune exhaustion in CHB patients with NAFLD. This preliminary finding indicates that, while no relationships between NAFLD associated metabolic risk factors and CHB specific immune responses were identified, patients with CHB and NAFLD may have increased T-cell immune exhaustion.

2.5.1. Limitations: The findings of this study provide an indication of how the presence of both CHB and NAFLD can affect the risk of liver fibrosis progression; however, several limitations must be considered. The current study did not find any effects of hepatic steatosis severity (based on CAP measurement) on HBV viral factors analyzed (i.e., HBV DNA, RNA, viral genotypes/variants, and quasispecies diversity). However, both NAFLD and CHB are complex chronic diseases that change over time. Therefore, additional longitudinal follow-up cohort studies may be needed to determine the causal relationships and the interactions between CHB and NAFLD. This study also relied on non-invasive methods of determining liver disease. As liver biopsy is the gold-standard in determining the degree of hepatocyte steatosis, fibrosis, and necroinflammatory activity, the non-invasive measures used in this study may not as accurately reflect the degree of liver disease. The majority of patients recruited were Asian, HBeAg negative, with genotype B or C infection which is generally reflected in the population HBV patients followed by the study investigators, but limits the generalizability to other CHB patients with NAFLD[121]. The PCR amplified regions of the preC/C and preS/S genes also do not cover the entirety of their respective genes, but rather encompassed an area with mutations of interest. Therefore, the genetic diversity analyses in this study

only represent a portion of the genes of interest and may not reflect the diversity in the entirety of their genes, or the whole HBV genome.

In this prospectively recruited, multi-ethnic cohort of NAFLD and CHB patients differences in novel HBV replication markers, viral diversity, and host cytokine patterns were found in association with metabolic risk factors. We found that the majority were HBeAg negative, showed low HBV S gene diversity, and a lower HBV specific antigen response. This data suggests that differences in the host antiviral immune response and viral replication markers in patients with NAFLD and CHB may influence liver disease risk. This is consistent with previous studies that have identified negative associations between NAFLD and CHB in patients with active HBV (HBsAg positive) infection, rather than past exposure (i.e., HBsAg clearance)[52]. The levels of proinflammatory cytokine levels did not correlate with hepatic inflammation / fibrosis or HBV replication, possibly indicating altered mechanisms for host immune control of HBV in patients with NAFLD and CHB.

Table 4: Summary of clinical and demographic data in HBeAg positive and HBeAg negative CHB patients with NAFLD.

Clinical/Demographic variable	HBeAg status		
	Negative	Positive	p
n	45	3	
Age	45.0 (9.7)	38.3 (18.9)	0.279
% Female	37.8	0	0.543
Ethnicity	27 SEA/7 SA/10 Afr/1 Cau	2 SEA/1 Cau	0.196
Mean log HBV DNA, copies/mL (SD)	3.4 (1.3)	8.8 (0.3)	0.004*
Mean log qHBsAg, pg/mL (SD)	2.8 (1.2)	4.7 (0.3)	0.020*
Mean log mRNA, copies/mL (SD)	1.0 (1.7)	2.6 (3.7)	0.689
Mean log pgRNA, copies/mL (SD)	0.4 (1.0)	2.7 (2.1)	0.014*
Mean CAP, dB/m (SD)	300.6 (52.4)	345.0 (21.3)	0.149
Mean LSM, kPA (SD)	5.8 (2.0)	4.9 (1.8)	0.383
Mean ALT, IU/mL (SD)	40.9 (25.9)	47.0 (10.4)	0.295
Mean BMI (SD)	28.6 (4.5)	26.3 (2.0)	0.446
% with obese waist circumference	68.6	50	0.956
% with metabolic syndrome	35.6	0	0.546

HBeAg – Hepatitis B virus E antigen (Abbot); SEA – South east Asian; SA - South Asian; Afr – African/Caribbean; Cau – Caucasian; HBV – hepatitis B virus; qHBsAg – quantitative hepatitis B virus surface antigen (Abbott); CAP – controlled attenuation parameter; LSM – liver stiffness measurement; BMI – body mass index. *p>0.05.

Table 5: Comparison of hepatic steatosis severity, as determined by controlled attenuation parameter (CAP) score, obesity and presence of metabolic syndrome between HBV genotypes.

HBV Genotype	n	% with CAP >280	% with metabolic syndrome	% Obese (based on BMI)
A	6	60	25	50
B	9	44.4	12.5	22.2
C	21	80	50	45.5
D	8	62.5	37.5	37.5
E	3	33.3	0	100

HBV – Hepatitis B virus; CAP – Controlled attenuation parameter; BMI – body mass index.

Table 6: Analysis of hepatitis B virus (HBV) markers and serum cytokine levels according to clinical and metabolic risk factors associated with non-alcoholic fatty liver disease (NAFLD). (Kruskal-Wallis).

HBV/Host variable	CAP median rank		Obese median rank		Metabolic syndrome (MS) median rank		Diabetes median rank	
	>280	<280	Obese	Non-obese	MS	No MS	Diabetic	Non-diabetic
qHBsAg	13.29	16.36	19.38	11.44	11.64	16.35	11.86	15.38
	p=0.335		p=0.012*		p=0.138		p=0.326	
HBV DNA	20.17	18.36	19.62	22.45	16.6	19.88	21.9	17.93
	p=0.628		p=0.449		p=0.360		p=0.321	
HBV mRNA	21.31	20.39	21.9	23.04	21	26.2	15.95	22.63
	p=0.787		p=0.728		p=0.810		p=0.076	
HBV pgRNA	21.72	21.1	25.38	20.92	19	21.4	18	21.97
	p=0.796		p=0.054		p=0.312		p=0.139	
S gene diversity	14.74	19.86	17.21	13.61	18.33	13.61	22.56	12.93
	p=0.142		p=0.913		p=0.150		p=0.008*	
C gene diversity	10.73	11.67	13.15	9.11	8.17	11.5	9.3	10.9
	p=0.755		p=0.150		p=0.247		p=0.600	
GM-CSF	9.82	11.33	8.1	12.9	10.33	8.27	10.6	8.33
	p=0.569		p=0.069		p=0.421		p=0.398	
IFN-γ	10.23	10.83	8.65	12.35	12.67	7	12.2	7.67
	p=0.819		p=0.161		p=0.027*		p=0.092	
IL-1β	9.55	11.67	8.65	12.35	8.67	9.18	7.6	9.58
	p=0.425		p=0.161		p=0.841		p=0.460	
IL-2	9.64	11.56	8.6	12.4	8.5	9.27	8	9.42
	p=0.470		p=0.150		p=0.762		p=0.597	
IL-4	10.73	9	8.5	11.35	8.75	8.35	8.2	8.64
	p=0.507		p=0.268		p=0.870		p=0.865	
IL-5	10	11.11	8.95	12.05	10.67	8.09	11.4	8
	p=0.675		p=0.240		p=0.314		p=0.205	
IL-6	10.64	10.33	10.55	10.45	9.67	8.64	8.6	9.17
	p=0.909		p=0.970		p=0.687		p=0.833	
IL-8	12.27	8.33	10.9	10.1	13.17	6.73	14	6.92
	p=0.138		p=0.762		p=0.012*		p=0.008*	
IL-10	8.32	13.17	12.7	8.3	10	8.45	8.8	9.08
	p=0.068		p=0.096		p=0.546		p=0.916	
IL-12	10.09	11	11.25	9.75	8.92	9.05	7.3	9.71
	p=0.719		p=0.551		p=0.958		p=0.343	
IL-13	9.64	11.56	10.15	10.85	10.58	8.14	10.7	8.29
	p=0.463		p=0.788		p=0.332		p=0.363	
MCP-1	10.91	10	8.7	12.3	10.67	8.09	11.4	8
	p=0.732		p=0.174		p=0.315		p=0.206	
TNFα	10.41	10.61	10.5	10.5	10.33	8.27	9.6	8.75
	p=0.939		p=1.000		p=0.421		p=0.752	

CAP – Controlled attenuation paramenter; qHBsAg – quantitative hepatitis B surface antigen (Abbott); HBV – hepatitis B virus; pgRNA – pregenomic RNA; S gene – Surface gene; C gene – Core gene; GM-CSF - Granulocyte-macrophage colony-stimulating factor; IFN-γ – interferon gamma; IL – interleukin; MCP – monocyte chemoattractant protein; TNFα – tumor necrosis factor alpha. *p < 0.05.

Table 7: Analysis of metabolic factors associated with non-alcoholic fatty liver disease (NAFLD), identified as predictors of viral and immune variables by Kruskal-Wallis test and multiple linear regression.

Dependent variable	Explanatory variable	Standardized coefficient	t	p
Diabetes	IL-8	-0.72	-4.06	0.002*
	S gene diversity	-0.32	-1.79	0.102
Metabolic syndrome	IL-8	-0.63	-2.86	0.014*
	IFN-γ	0.01	0.03	0.973
Dyslipidemia	IL-8	-0.44	-1.74	0.106
	IFN-γ	0.15	0.59	0.568
Hypertension	IL-8	-0.37	-1.41	0.183
	IFN-γ	-0.03	-0.1	0.919
Waist circumference	IL-8	-0.27	-1.08	0.298
	IFN-γ	0.4	1.59	0.137
Obese (BMI)	qHBsAg	-0.48	-2.88	0.008*

IL-8 – Interleukin 8; S gene – surface gene; IFN-γ – interferon gamma; BMI – body mass index; qHBsAg – quantitative hepatitis B surface antigen. *p < 0.05.

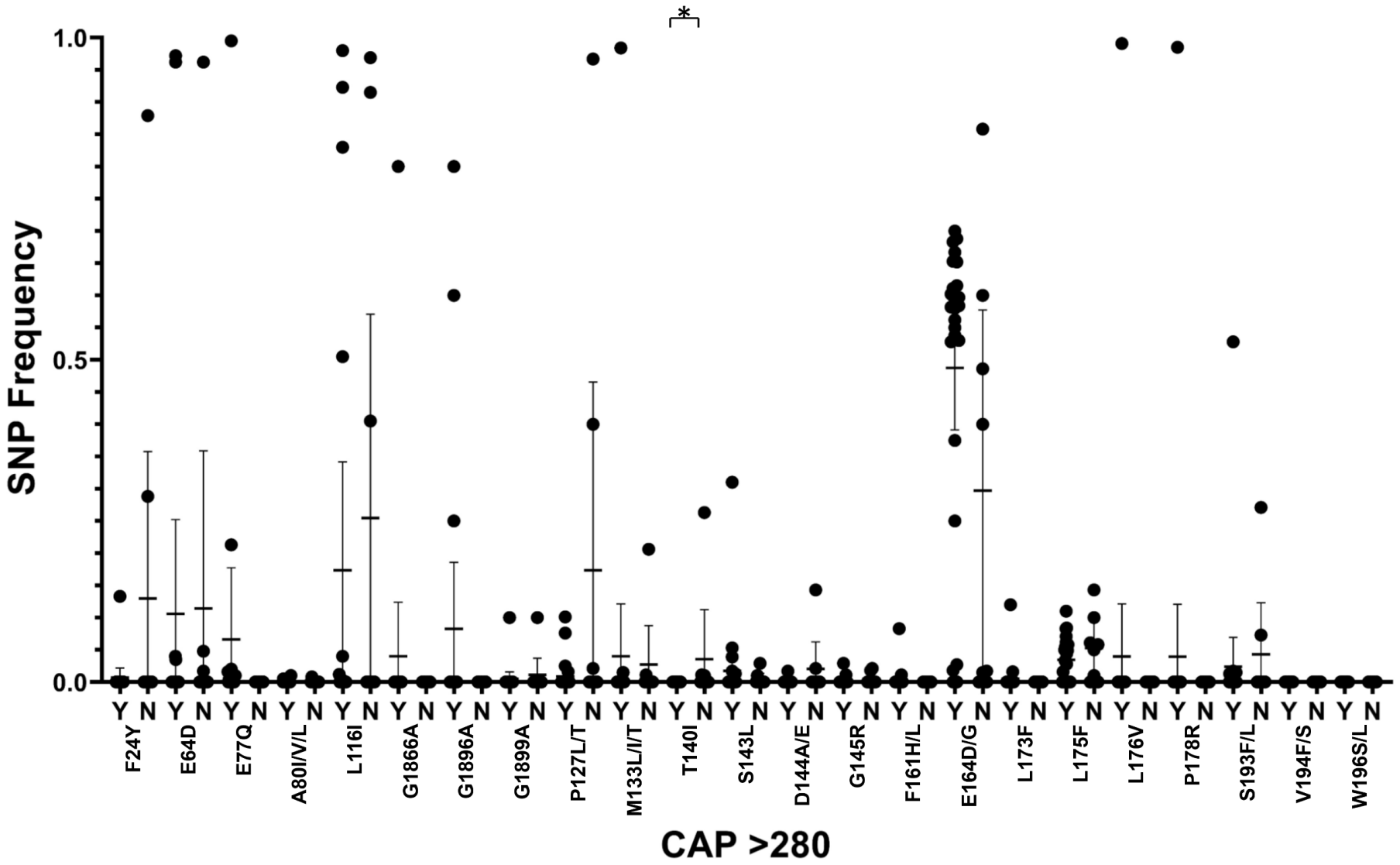

Figure 3A: Frequency of mutations in the HBV preCore (C)/C and preSurface (S)/S genes at sites relating to increased liver disease progression, hepatitis B E antigen negativity, immune escape, and anti-viral drug resistance by controlled attenuation parameter (CAP) score. SNP – Single nucleotide polymorphism; Y - Yes; N – No. *significant difference, p>0.05.

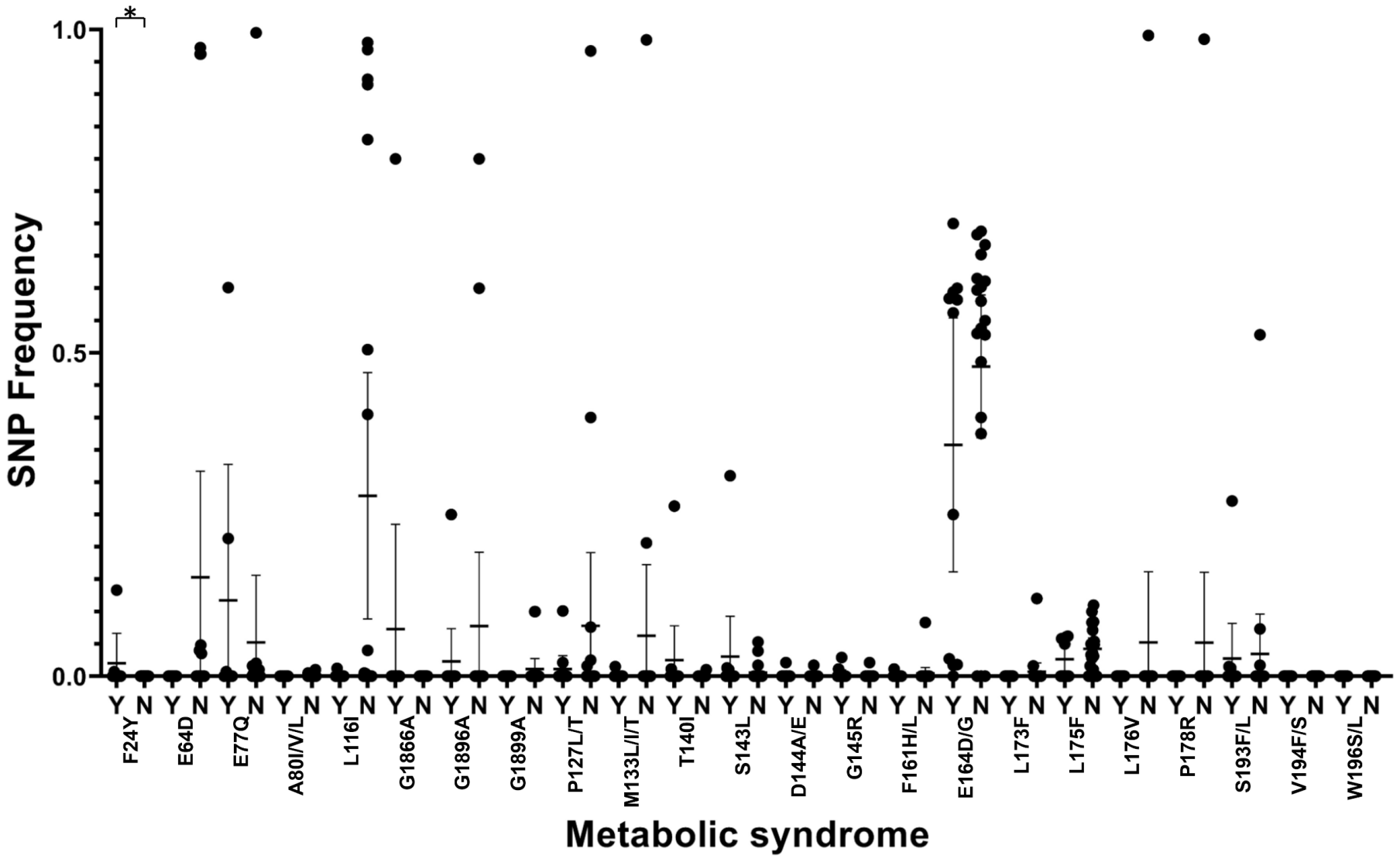

Figure 3B: Frequency of mutations in the HBV preCore (C)/C and preSurface (S)/S genes at sites relating to increased liver disease progression, HBV E antigen negativity, immune escape, and anti-viral drug resistance based on the diagnosis of metabolic syndrome. SNP – Single nucleotide polymorphism; Y – Yes; N – No. *significant difference, p>0.05.

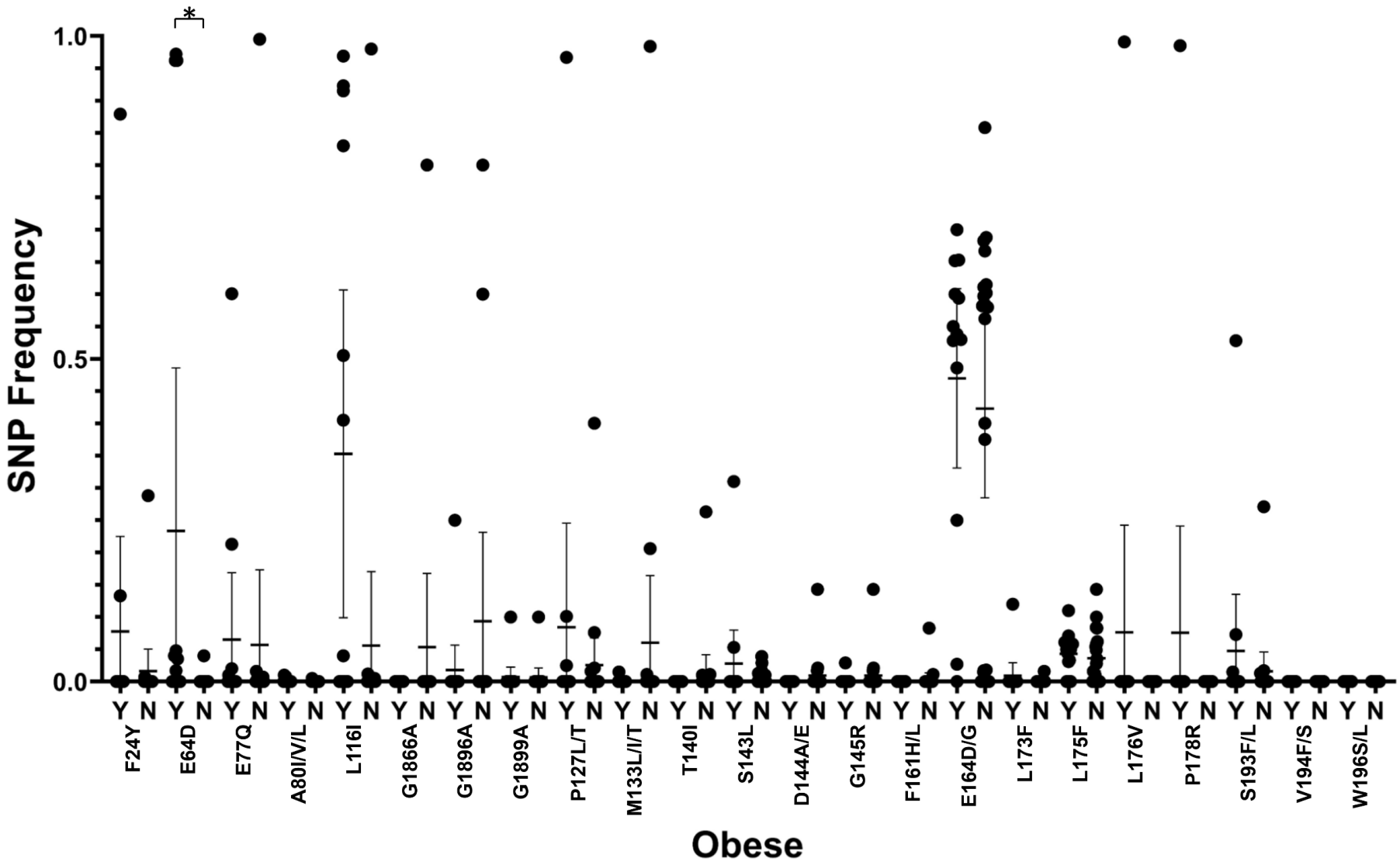

Figure 3C: Frequency of mutations in the HBV preCore (C)/C and preSurface (S)/S genes at sites relating to increased liver disease progression, HBV E antigen negativity, immune escape, and anti-viral drug resistance by obesity status based on body mass index (BMI). SNP – Single nucleotide polymorphism; Y – Yes; N – No. *p > 0.05.

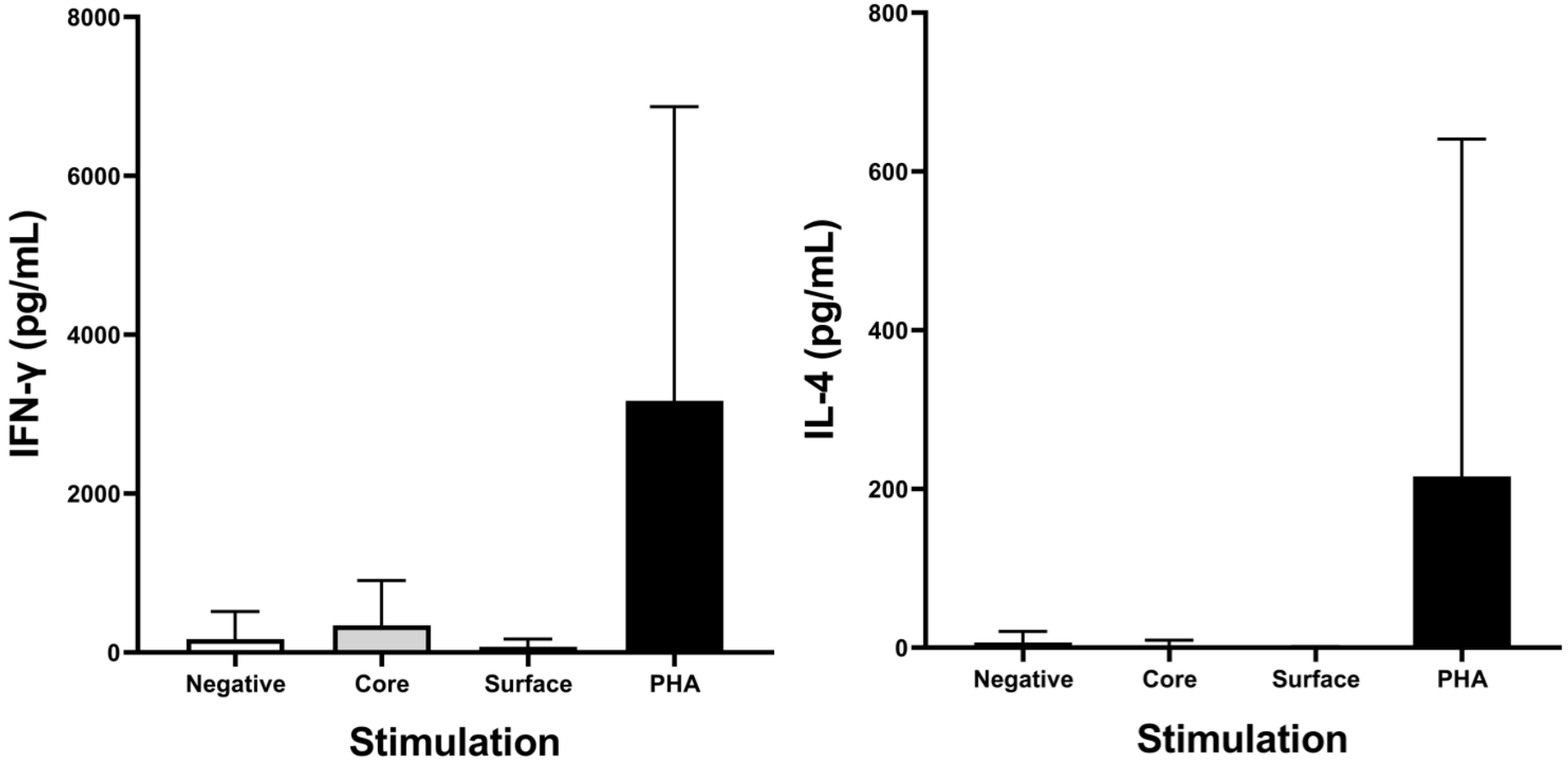

Figure 4: Comparison of IFN-γ and IL-4 concentrations in supernatant from unstimulated (negative) PBMCs, or PBMCs stimulated by HBV core or surface antigen vs. Phytohaemagglutinin (PHA).

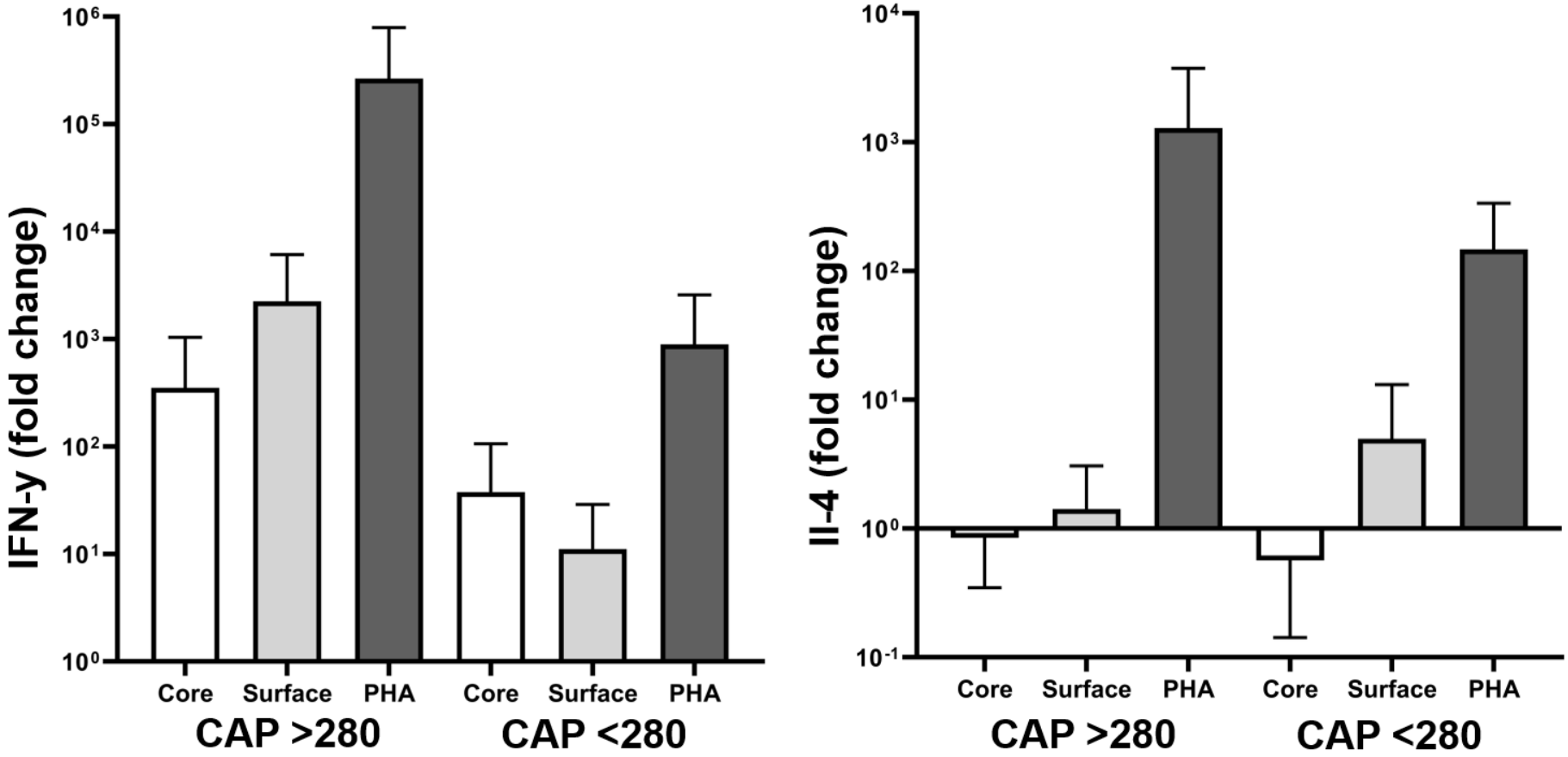

Figure 5A: Comparison of fold change in IFN-γ and IL-4 supernatant concentrations by PBMCs stimulated by HBV core or surface antigen, or Phytohaemagglutinin (PHA) in subjects with CHB and NAFLD, stratified by high or low controlled attenuation parameter (CAP) score (i.e., severity of hepatic steatosis) compared to unstimulated controls.

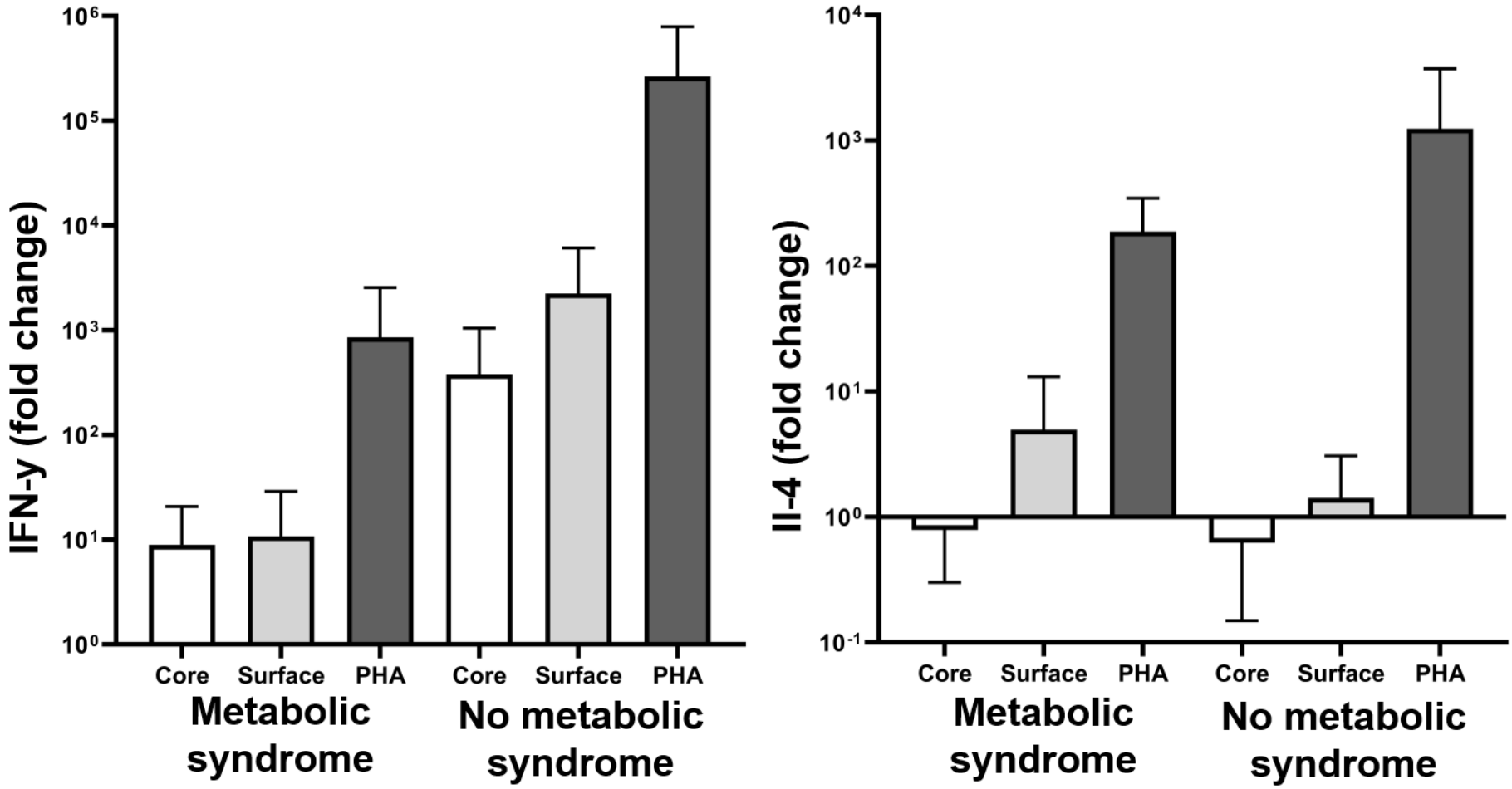

Figure 5B: Comparison of fold change in IFN-γ and IL-4 supernatant concentrations from PBMCs stimulated with HBV core or surface antigen vs. Phytohaemagglutinin (PHA) in subjects with CHB and NAFLD with or without the metabolic syndrome compared to unstimulated controls.

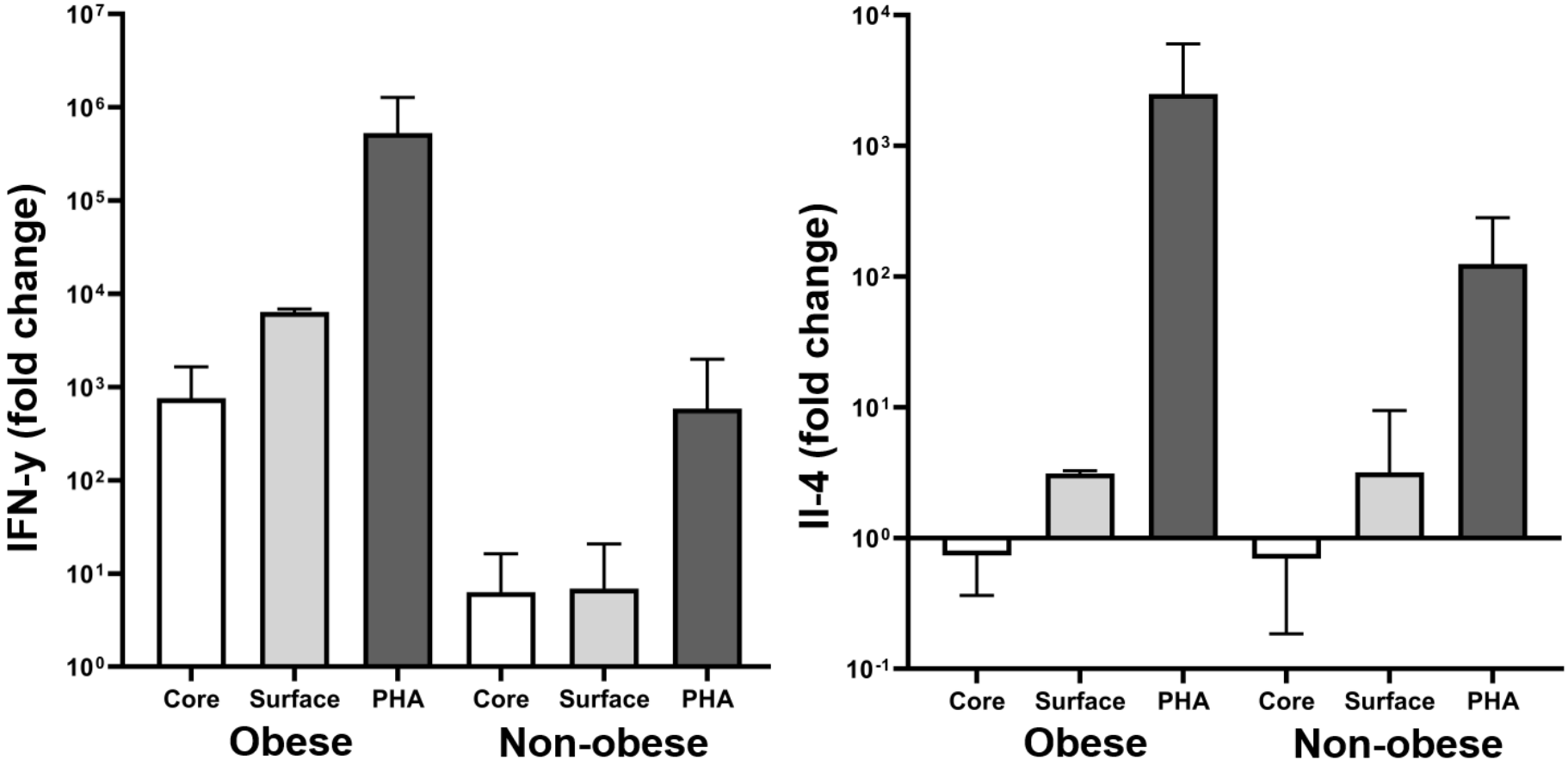

Figure 5C: Comparison of fold change in IFN-γ and IL-4 in supernatant of PBMCs stimulated by HBV core or surface antigen, or Phytohaemagglutinin (PHA) in subjects with CHB and NAFLD based on body mass index classification (obese or non-obese) compared to unstimulated controls.

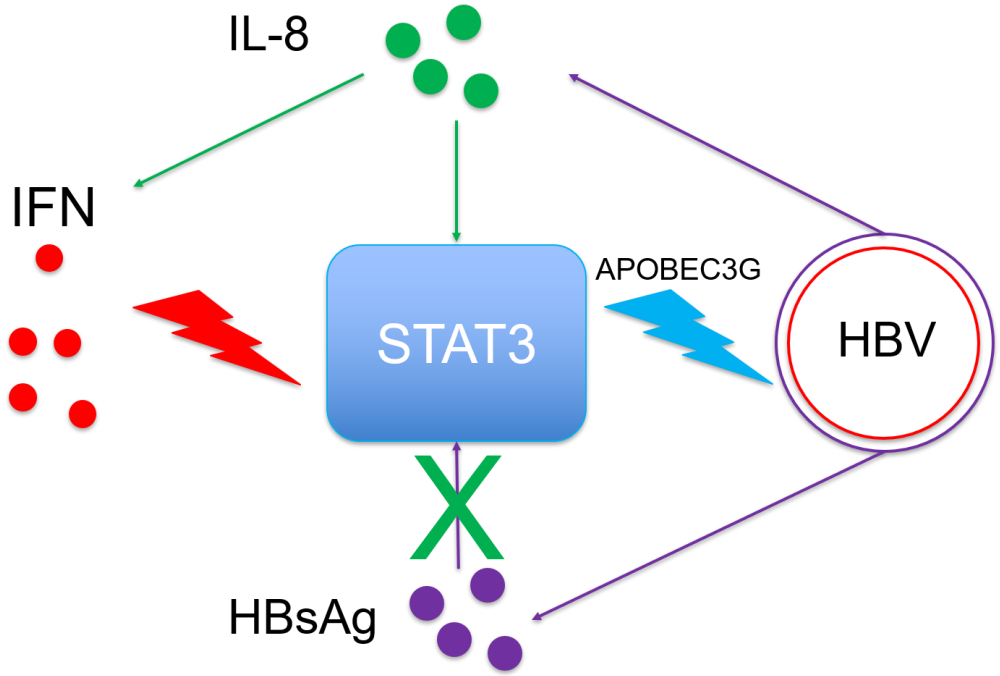

Figure 6: Proposed mechanism for the interaction of IL-8, HBV surface antigen (HBsAg) and, interferons (IFN). Type I and II IFNs regulate APOBEC3G via the STAT3 pathway. However, HBsAg has been found to prevent the upregulation of APOBEC3G through interaction with STAT3. Therefore, IFN responses to infection may cause a selective pressure on HBV to select for HBsAg that can interact with the STAT3 pathway. IL-8 has been shown to be upregulated in both NAFLD and HBV, interact with STAT3, and decrease the sensitivity of HBV to IFNs. Therefore, the upregulation of IL-8 in NAFLD could reduce the selective pressure of IFN on HBV, allowing for viable HBsAg phenotypes that may not interact with the STAT3 pathway.

Chapter 3: Discussion

Previous large scale epidemiological studies have identified relationships between CHB and NAFLD and focused on identifying NAFLD prevalence and incidence in CHB. Several studies have looked at the biological relationships between CHB and NAFLD, but have primarily analyzed only a single clinical variable associated with NAFLD, such as hepatic steatosis or insulin resistance. One study has evaluated the relationship between HBV and metabolic factors associated with NAFLD in patients with CHB and NAFLD. Hui *et al.* conducted a prospective case-control study where non-invasive measures of liver disease were used to assess the relationship between the degree of hepatic steatosis and HBV viral load (HBV DNA levels) in patients with NAFLD[62]. Although, NAFLD associated metabolic risk factors were included in their analysis, it only identified the effects on HBV DNA and did not observe any other markers of viral replication, viral genotype or host immune markers. Therefore, the study presented in this thesis provides novel data on the relationship between NAFLD and CHB in patients using a more comprehensive range of HBV and NAFLD biomarkers. In this thesis, patient NAFLD associated metabolic risk factors were compared to HBV replication markers, viral genetic factors, and host cytokine patterns and immune responses. In CHB patients with metabolic syndrome and diabetes, inflammatory immune markers and HBV viral diversity were increased. Additionally, obese patients were found to have increased qHBsAg levels.

The innate immune response seen in NAFLD appears to be a major mechanism for the interaction between HBV and NAFLD, and several possible mechanistic pathways could be further explored. The APOBEC3G could be influenced by IL-8 to

diminish the effects of IFN-mediated host antiviral responses in NAFLD patients with metabolic syndrome, potentially resulting in increased S gene diversity. The potential influence of IL-8 in the pathogenesis of CHB and NAFLD patients be could be clarified by measuring the activation of APOBEC3G by IFNs, and its relation to HBV genetic and clinical characteristics. Other studies have found that several host factors such as the gut microbiome, play a major role in the immune response to HBV. The gut microbiota have been linked to HBV seroconversion and treatment responses[23,24]. Intestinal bacterial dysbiosis, and its contributions to the NAFLD inflammatory state, has been well established in NAFLD pathogenesis and has been linked to every NAFLD clinical (metabolic risk) factor[37]. Therefore, there is likely a role for the gut microbiota in regulating the inflammatory state in CHB and NAFLD. Future analyses of the intestinal microbiome of patients with comorbid CHB and NAFLD would help identify potential methods for interventions to reduce immune inflammation and liver disease. However, human microbiome sampling procedures often rely on direct fecal sampling, which biases the bacterial populations found towards those within the colon, or colonoscopy, which is invasive test[122]. In this regard, mouse models to study the effects of the microbiome in NAFLD and CHB mice would provide further clarification of the effects of dysbiosis of bacterial species.

There is conflicting evidence on the relationships between CHB and NAFLD. In a recent meta-analysis, Xiong *et al.* confirmed that the risk of NAFLD was decreased in patients with CHB[50]. However, published studies have demonstrated conflicting evidence with respect to the relationship between NAFLD and markers of CHB activity, disease clearance, and progression. Additionally, there is varying evidence on the

effects of NAFLD associated metabolic risk factors on HBV antiviral drug efficacy, and the incidence of NAFLD in CHB. One relationship that has been well established in patients with CHB and NAFLD is the increased chance of developing HCC. However, this relationship could also depend on specific NAFLD associated metabolic risk factors. The identification of long-term trends in disease progression for individuals with NAFLD and CHB would enhance understanding of the disease interactions. As an extension of the project presented in this thesis, patients will continue to be monitored and sampled during regular follow-up visits to the sites of recruitment. Continued recruitment and long-term data collection could allow for more nuanced analyses of HBV genetic diversity and mutations through the identification of changes of patterns in viral quasispecies. Additionally, the effects of individual NAFLD associated metabolic risk factors on liver disease progression can be identified. Further recruitment and follow-up of patients will also provide additional power to future statistical analyses and confirm these associations.

One of the major applications of research on the interactions between CHB and NAFLD is the development of clinical guidelines for the treatment of patients with both diseases. Two studies have identified decreased responses to entecavir treatment for CHB in Chinese populations with NAFLD[87,89]. However, this relationship is contested by two Turkish studies that found no differences between entecavir responses in NAFLD and non-NAFLD patients[85,86]. While mutations associated with entecavir drug resistance were found in patients in the current study, these were generally found in low proportions and likely would not affect entecavir treatment responses. Furthermore, phenotypic tests of drug resistance would be necessary to confirm the viral strain's

resistance to entecavir, and it is acknowledged that overall the reported risk of entecavir resistance in treatment naïve patients is very rare (<1-2%)[4]. While these mutations were not significantly associated with differences in respect to the presence or absence of metabolic risk factors in NAFLD, previous studies have established that IL-8 decreases the susceptibility of HBV to IFN[116]. As demonstrated in this study, IL-8 is increased in CHB and NAFLD patients with metabolic syndrome and diabetes. This is consistent with the findings of Gong *et al.* who identified a decreased response to pegylated-IFN therapy in patients with liver steatosis and insulin resistance[88]. However, there are several side effects associated with pegylated-IFN that also greatly reduce its use in clinical settings. Therefore, the recommended treatments for CHB and NAFLD patients likely would not be different than patients with only CHB.

In the current study, we noted an increase in S gene diversity in patients with diabetes. Increased antigen diversity has been previously associated with seroconversion in HBV, and NAFLD is associated with increased HBsAg seroclearance and seroconversion in mice[24]. However, serum HBsAg levels were increased in obese patients. This suggests that in NAFLD, diabetic patients may be more prone to HBsAg seroconversion and immune control of the virus, while obesity may be linked to poorer control. Therefore, the study reinforces current clinical guidelines regarding the importance of early diet and lifestyle intervention in obese CHB patients to reduce the risk of liver disease progression.

NAFLD is a complex disease that impacts HBV-related liver disease progression and *vice versa*. In this thesis, we found differences in the viral genome, replication and host immune patterns in patients affected by both diseases. The study contributes to

accumulating evidence on the complex interplay between both diseases and will inform future research studies to improve the management of patients living with chronic hepatitis B infection.

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Appendix A: Supplementary tables

Table S1: Primers used in PCR reactions.

HBV region amplified	Primer/probe name	Sequence (5' to 3')	HBV primer binding site
preCore (C)/C	preCC DF	GGATGGAGACCACCGTGAACG	nt1606-1626
preC/C	preCC DR	GAGGGAGTTCTTCTTCTAGG	nt2366-2385
preC/C nested	preCC NF	TCACCTCTGCCTAATCATC	nt1825-1843
preC/C nested	preCC NR	GGAGTGCGAATCCACACTCC	nt2267-2286
preSurface (S)/S	HBPr134	TGCTGCTATGCCTCATCTTC	nt414-433
preS/S	HBPr135	CARAGACARAAGAAAATTGG	nt803-822
preS/S nested	HBPr75	CAAGGTATGTTGCCCGTTTGTCC	nt455-477
preS/S nested	HBP94	GGYAWAAAGGGACTCAMGATG	nt775-795
preS/S NGS	HBPr75 w/ adapter	TCGGCAGCGTCAGATGTGTATAAGAGAC AGCAAGGTATGTTGCCCGTTTGTCCTCG	nt455-477
preS/S NGS	HBP94 w/ adapter	GTCTCGTGGGCTCGGAGATGTGTATAAG AGACAGGGYAWAAAGGGACTCAMGATG	nt775-795
preC/C NGS	HBPr7s w/ adapter	TCGGCAGCGTCAGATGTGTATAAGAGAC AGGAATTTGGAGCTWCTGYGGAG	nt1922-1943
preC/C NGS	Core-NGSn w/ adapter	GTCTCGTGGGCTCGGAGATGTGTATAAG AGACAGCCACACYCCRAARGASACCA	nt2266-2286
Plasmid Cloning	M13 F	GTAAAACGACGGCCAGT	Binds to T-Easy plasmid
Plasmid Cloning	M13 R-17	CAGGAAACAGCTATGAC	Binds to T-Easy plasmid
Total HBV RNA	Anchored Long Primer	ACCACGCTATCGCTACTCAC GAGAGTAGCGATAGCGTGGT	NA
Total HBV RNA	HBV X Long Primer	CAACTTTTTCACCTCTGCCTA	nt1817-1837
Total HBV RNA	HBV X Long Probe	FAM - CATGTCCYACTGTTCAAGCCTCCAAG	nt1851-1876
HBV pgRNA	FWD Primer	GGAGTGTGGATTCGCACTCCT	nt2267-2287
HBV pgRNA	REV Primer	AGATTGAGATCTTCTG CGAC	nt2421-2440
HBV pgRNA	Probe	FAM - AGGCAGGTCCCCTAGAAGAA GAACTCC	nt2365-2391

Table S2: PCR thermocycler protocols used for conventional PCR.

Amplified gene	PCR primers used	Thermocycler protocol
preCore (C)/C	preCC DF preCC DR preCC NF preCC NR	94ºC - 4 minutes 40 Cycles of: 94ºC - 30 seconds 55ºC - 1.5 minutes 72ºC - 1.5 minutes Followed by: 72ºC - 10 minutes 4ºC hold
preSurface (S)/S	HBPr134 HBPr135 HBPr75 HBP94	94ºC - 4 minutes 40 Cycles of: 94ºC - 30 seconds 45ºC - 30 seconds 72ºC - 30 seconds Followed by: 72ºC - 10 minutes 4ºC hold
NGS C and S	HBPr75 w/ adapter HBP94 w/ adapter HBPr7s w/ adapter Core-NGSn w/ adapter	94ºC - 4 minutes 25 Cycles of: 94ºC - 15 seconds 55ºC - 30 seconds 72ºC - 4 seconds Followed by: 4ºC hold

Table S3: Non-Caucasian ethnicity specific obesity cut-offs for BMI and waist circumference.

Reference	Ethnicity	Variable	Sex	Obesity cut-off
Tillin, 2015[110]	African/Caribbean	Waist circumference	M	90.6
			F	81.2
		BMI	M	27.2
			F	27.2
	South East Asian	Waist circumference	M	90.4
			F	84.0
		BMI	M	25.2
			F	25.2
Misra, 2009[111]	East Indian	Waist circumference	M	90
			F	80
		BMI	M	25
			F	25

Table S4: Clinically relevant mutations in the HBV genome by gene.

Gene	Mutation ID	Phenotype associated with mutation
preCore/Core	G1862T	Inhibited HBeAg expression
preCore/Core	G1896A	Inhibited HBeAg expression
preCore/Core	G1899A	Inhibited HBeAg expression
preCore/Core	F24Y	Increased cirrhosis and HCC development
preCore/Core	E64D	Increased cirrhosis and HCC development
preCore/Core	E77Q	Increased cirrhosis and HCC development
preCore/Core	A80I/V/L	Increased cirrhosis and HCC development
preCore/Core	L116I	Increased cirrhosis and HCC development
preSurface/Surface	P127L/T	Immune escape phenotype
preSurface/Surface	M133L/I/T	Immune escape phenotype
preSurface/Surface	T140I	Immune escape phenotype
preSurface/Surface	S143L	Immune escape phenotype
preSurface/Surface	D144A/E	Immune escape phenotype
preSurface/Surface	G145R	Vaccine escape phenotype
preSurface/Surface	F161H/L	Entecavir resistance
preSurface/Surface	E164D/G	Immune escape phenotype
preSurface/Surface	L173F	Lamivudine resistance
preSurface/Surface	L175F	Entecavir resistance
preSurface/Surface	L176V	Entecavir resistance
preSurface/Surface	P178R	Immune escape phenotype
preSurface/Surface	S193F/L	Entecavir resistance
preSurface/Surface	V194F/S	Entecavir resistance
preSurface/Surface	W196S/L	Tebivudine resistance

Adapted from: Zhang, Z. H. *et al.* Genetic variation of hepatitis B virus and its significance for pathogenesis. *World J. Gastroenterol.* 22, 126–144 (2016).

Appendix B: Footnotes

Clinical data was missing for CAP scores (n=5) and waist circumference (n=12).

Appendix C: Additional methods

I. Protocol for the processing of blood samples:

1. Samples are spun down at 2000g for 10 minutes.
2. Serum and plasma supernatants are aliquoted and frozen at -20^{o}C for 2 hours before being moved to -80^{o}C for storage.
3. The remaining blood sample is pipetted into a 50 mL falcon tube. DPBS (ThermoFisher Scientific, Waltham, Massachusetts, USA) is added to the tube to double the volume of the blood.
4. 8 mL of diluted blood are slowly added to a 15 mL falcon tube containing 4mL of ficoll®-Paque Plus (GE Healthcare, Chicago, Illinois, USA) to create a floated layer.
5. The Ficoll-blood float is centrifuged at 2000 rpm for 20 minutes, with minimum deceleration to fractionate the blood.
6. A buffy coat layer is extracted using a Pasteur pipette and added to a 15 mL falcon tube, which is diluted up to 14 mL by DPBS (ThermoFisher Scientific, Waltham, Massachusetts, USA).
7. The 15 mL tube is then centrifuged at 2000rpm for 10 minutes. Supernatant is decanted and the pellet is resuspended in ACK lysis buffer (ThermoFisher Scientific, Waltham, Massachusetts, USA) and incubated at 37^{o}C for 10 minutes.
8. DPBS (ThermoFisher Scientific, Waltham, Massachusetts, USA) is added up to 14 mL and centrifuged at 2000 rpm for 10 minutes. Any supernatant is decanted.

9. The PBMC pellet is resuspended in 1 mL of DPBS and the PBMC concentration and viability is measured using a Countess™ Automater Cell Counter (Life Technologies, Carlsbad, California, USA) device.
10. The PBMCs are diluted with DPBS (ThermoFisher Scientific, Waltham, Massachusetts, USA) to 14 mL and centrifuged at 2000 rpm for 10 minutes. PBMCs are decanted and resuspended in fetal bovine serum (FBS, ThermoFisher Scientific, Waltham, Massachusetts, USA) with 10% DMSO (Sigma Aldrich, St. Louis, Missouri, USA) and frozen overnight -80^{o}C before being added to liquid nitrogen for storage.

(II) Protocol for the isolation of total DNA from plasma:

1. 500 uL plasma is added to 500 uL PBS (ThermoFisher Scientific, Waltham, Massachusetts, USA) in a 2 mL tube.
2. 1 mL phenol (Sigma Aldrich, St. Louis, Missouri, USA) is added to the tube and is mixed and incubated at 50^{o}C for 10 minutes. Tubes are centrifuged at 13000g for 10 minutes.
3. The aqueous top layer of the supernatant is removed and added to a new tube. Step 2 is repeated.
4. The aqueous top layer of the supernatant is removed and added to a new tube. An equal volume of phenol:chloroform:isoamyl alcohol 25:24:1 (Sigma Aldrich, St. Louis, Missouri, USA) is added to the tube. Tubes are centrifuged at 13000g for 10 minutes.
5. Step 4 is repeated.

6. The aqueous top layer of the supernatant is removed and added to a new tube. An equal volume of chloroform (Sigma Aldrich, St. Louis, Missouri, USA) is added to the tube. Tubes are centrifuged at 13000g for 10 minutes.
7. The aqueous top layer of the supernatant is removed and added to a new tube. 2.5 volumes of 100% ethanol and 0.1 volumes of 5M sodium acetate (Sigma Aldrich, St. Louis, Missouri, USA) are added to the tube.
8. Tubes are stored overnight at -80^{o}C to precipitate DNA.
9. Tubes are centrifuged at 14000g for 30 minutes at 4^{o}C. Supernatant is decanted and the DNA pellet is washed in 700 uL of 70% ethanol.
10. Tubes are centrifuged at 14000g for 30 minutes at 4^{o}C. Supernatant is decanted and the pellet is dried for 1 hour at room temperature.
11. The DNA pellet is dissolved in 30uL pure DNA/RNA free H_2O (ThermoFisher Scientific, Waltham, Massachusetts, USA) and is stored at -20^{o}C.

If HBV DNA could not be amplified from the sample, then the direct and nested PCR was repeated using the remaining volume (25 µL) of the extracted DNA suspension as the direct PCR round template. If no detectable HBV DNA could be detected from a single plasma extraction, 4mL of plasma were concentrated by centrifugation at 130,000g for 18 hours with 500µL of the lowest fraction being used for HBV DNA isolation using the same phenol-chloroform extraction protocol.

(III) Protocol for HBV total RNA extraction from plasma:

1. Add 500uL plasma and 1mL Trizol (Sigma Aldrich, St. Louis, Missouri, USA) to a 2mL microcentrifuge tube. Incubate samples at room temperature for 5 minutes.

2. Add 200uL chloroform (Sigma Aldrich, St. Louis, Missouri, USA) to each sample and shake vigorously for 15 seconds. Incubate samples at room temperature for 3 minutes.
3. Centrifuge the samples at 12,000g for 15 minutes at 4^{o}C. Transfer the aqueous top layer of the supernatant to a new tube.
4. Add 500uL of 100% isopropanol (Sigma Aldrich, St. Louis, Missouri, USA) and incubate at room temperature for 10 minutes.
5. Centrifuge the samples at 12,000g for 10 minutes at 4^{o}C. Decant the supernatant.
6. Wash the RNA pellet with 1mL of 75% ethanol. Invert the samples to mix.
7. Centrifuge the samples at 7,500g for 5 minutes at 4^{o}C. Decant supernatant.
8. Dissolve the pellet in 30uL of pure DNA/RNA free H_2O (ThermoFisher Scientific, Waltham, Massachusetts, USA).

Following RNA extraction, samples are converted to complementary (c)DNA for measurement using qPCR. Two methods of cDNA conversion are used in this study. Rapid amplification of complimentary ends (RACE) cDNA conversion involves using the DNA polymerase to amplify copies of cDNA only between a single point of interest and the 3' or 5' end. The HBV X gene contains two polyadenylation sites that comprises the 3' end of HBV mRNA transcripts. Using primers that bind to the 3' polyadenylated tail of the X gene mRNA, RACE cDNA conversion can produce a cDNA product that can be measured using qPCR to estimate the total amount of viral mRNA. cDNA reverse transcription for RACE RNA uses the qScript™ cDNA synthesis kit (Quanta Biosciences Inc, Gaithersburg, Maryland). The pgRNA cDNA is formed during a procedure for total

RNA synthesis and is also quantified using qPCR. Total RNA reverse transcription uses the SuperScript® II reverse transcriptase kit (Life Technologies, Carlsbad, California, USA). Plasmid standards with concentrations from 10^7-10^0 copies/uL are used as positive controls for both pgRNA cDNA and RACE cDNA quantification.

(IV) Protocol for HBV clonal preparation and sequencing:

1. PCR products are inserted into a pGEM®-T Easy plasmid vector (Promega, Madison, Wisconsin, USA) by incubating overnight at 4^{o}C according to manufacturer protocols.
2. Combine 2uL plasmid vector with 100uL chemically competent *E. Coli* cells. Incubate on ice for 30 minutes. Heat shock the bacterial solution at 42^{o}C for 1 minute and then incubate on ice for 2 minutes.
3. Add 200uL LB broth to the bacterial solution and incubate at 37^{o}C for 1 hour.
4. Plate 100uL of bacterial solution on LB agar plates with 50ug/mL ampicillin (ThermoFisher Scientific, Waltham, Massachusetts, USA) and 200uL of ug/mL X-gal (ThermoFisher Scientific, Waltham, Massachusetts, USA). Incubate overnight at 37^{o}C.
5. Pick 10 bacterial colonies using blue/white screening.
6. Confirm the presence of the insert using PCR with primers specific to the inserted gene.
7. Add insert positive colonies to 2 mL of LB broth with 50 ug/mL ampicillin (ThermoFisher Scientific, Waltham, Massachusetts, USA). Incubate overnight at 37^{o}C.

8. The plasmid DNA is then extracted using a commercial GenElute™ Plasmid Miniprep kit (Sigma Aldrich, St. Louis, Missouri, USA) and manufacturer protocols.
9. All extracted plasmid samples are then sent for sequencing at an internal facility.

All sequencing alignments are performed using MEGA 7 software. All sequences were aligned using a series of 37 reference sequences with varying HBV genotypes (A-H) from several different countries. A gap opening penalty of 15 and gap extension penalty of 6.66 were used in the alignment. HBV genotype for each sample was confirmed using the NCBI database, sequence blast, and using phylogenetic clustering with the reference sequences. Between and within-group genetic diversity analyses for each clonal population are performed using a 1000 replication bootstrap Kimura 2-parameter model with pairwise deletion.

(V) Protocol for PBMC stimulation:

1. Thaw PBMCs in 37^{o}C water bath for 5 minutes.
2. Centrifuge the PBMCs at 200g for 20 minutes.
3. Decant the PBMC pellet and resuspended in 1 mL of DPBS and the PBMC concentration and viability is measured using a Countess™ Automater Cell Counter (Life Technologies, Carlsbad, California, USA).
4. Centrifuge the PBMCs at 200g for 20 minutes.
5. Decant the PBMC pellet and resuspend in 1 mL of RPMI media (ThermoFisher Scientific, Waltham, Massachusetts, USA) with 10% FBS (ThermoFisher

Scientific, Waltham, Massachusetts, USA). Dilute to a concentration of 1,000,000 cells/mL using RPMI media with 10% FBS.

6. Seed cell culture wells with 1 mL of PBMCs and incubate for 24 hours at 37^{o}C.
7. Add HBsAg, HBcAg, or Phytohemagglutinin (PHA, a plant lectin protein that is used to selectively stimulate T-cell responses[123], Merck & Co., Kenilworth, New Jersey, USA) to a concentration of 5ug/mL, mix well and incubate for 72 hours at 37^{o}C.
8. Transfer PBMC solution to tubes and centrifuge at 2000g for 20 minutes.
9. Collect supernatant and store at -80^{o}C.

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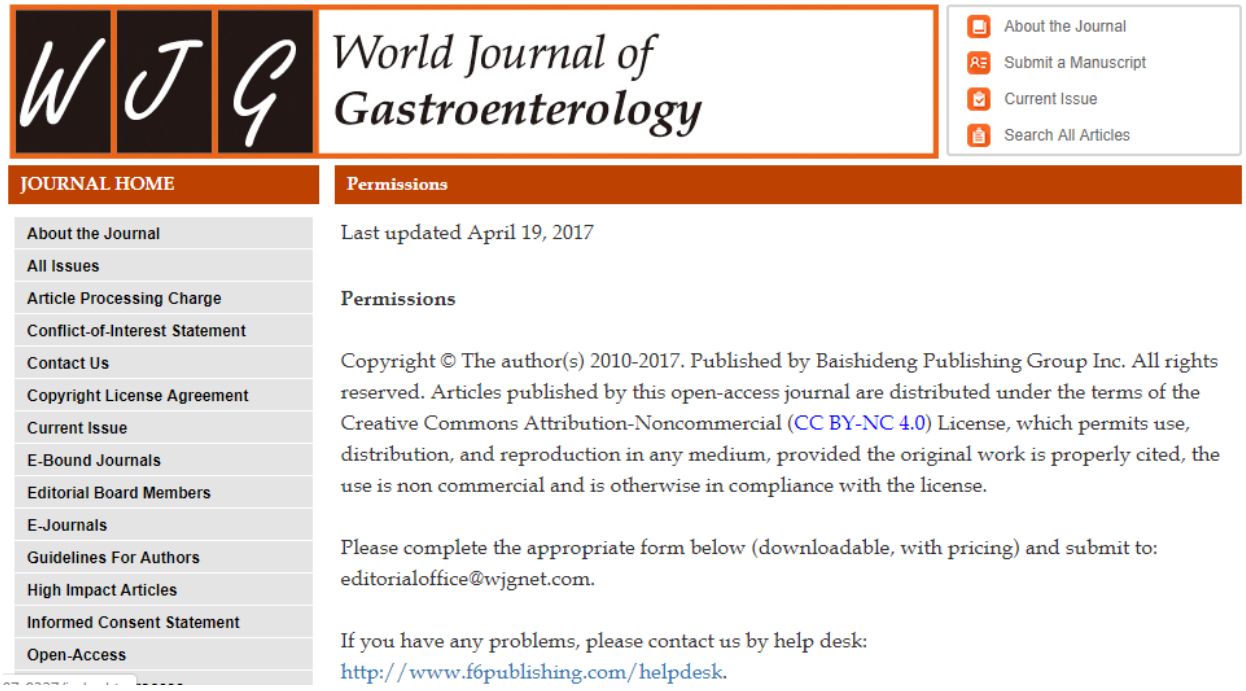

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