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ORIGINAL ARTICLE

Assessment of HBV variants and novel viral and immune biomarkers in chronic hepatitis B patients with metabolic dysfunction associated steatotic liver disease

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Abstract

Co-existing chronic hepatitis B virus (CHB) infection and metabolic dysfunction associated steatotic liver disease (MASLD) can exert complex effects on hepatic metabolism, requiring mechanistic study. CHB participants were assessed for MASLD and the impact of hepatic steatosis/metabolic syndrome (MetS) on novel viral and immunological markers. In this prospective, cohort study, untreated CHB subjects were assessed for liver disease by non-invasive tests (i.e. FibroScan, controlled attenuation parameter, CAP). Subjects were tested for cytokines and IFN-γ ELISPOT assay to HBV Surface (S) and Core (C) proteins. Standard HBV serological, exploratory biomarkers and deep sequencing of HBV S and C genes were performed. In 53 subjects (median age 45 years [SD = 10.6], 35% F, 56% Asian, 20% Black, 3% White), 94% (50) HBeAg negative, 63% genotype B/C, mean HBV DNA 3.2 $\log_{10}$ IU/mL (SD = 1.8), quantitative HBsAg 2.9 $\log_{10}$ IU/mL (SD = 1.2) and HBV pgRNA 2.1 $\log_{10}$ copies/mL (SD = 1.3). In enrolled subjects, the mean ALT was 41.9 U/L (SD = 24.0), FibroScan was 5.7 kPa (SD = 1.9) and CAP was 306.4 dB/m (SD = 49.0). The mean BMI was 28.2 kg/m^2 (SD = 4.2), 20% (11/53) had diabetes, 35% (19/53) dyslipidaemia and 24% (13/53) hypertension. Subjects with MetS and steatosis showed lower HBV markers ($p < .01$), higher HBV S diversity ($p = .02$) and greater frequency of HBV variants associated with host-anti-viral immune escape. Pro-inflammatory cytokine levels and HBV-specific cellular responses were higher in participants with hepatic steatosis. In CHB, MASLD/hepatic steatosis was associated with HBV variants and systemic immune responses potentially impacting liver disease progression despite low-level viraemia.

KEYWORDS
biomarkers, chronic hepatitis B, cytokines, metabolic dysfunction associated steatotic liver disease, T-cell response

Abbreviations: ALT, alanine transaminase; anti-HBc, antibody to HBV core; anti-HBs, antibody to HBV surface; BMI, body mass index; CAP, controlled attenuation parameter; CHB, chronic hepatitis B; HBeAg, HBV E antigen; HBsAg, HBV surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; LSM, liver stiffness measurement; MASLD, metabolic dysfunction associated steatotic liver disease; MetS, metabolic syndrome; mRNA, messenger RNA; NGS, next-generation sequencing; NRAg, HBV nucleic acid-related antigen; pgRNA, pre-genomic RNA; qAHBc, quantitative anti-hepatitis B core; qHBsAg, quantitative HBsAg; RACE, rapid amplification of complementary ends.

1 | INTRODUCTION

Changes in lifestyle, diet and physical activity patterns over the past few decades have contributed to a rise in the prevalence of obesity (i.e. from estimated 15–20% in 2005 to 30–32% in 2022) and the risk of metabolic dysfunction associated steatotic liver disease (MASLD, formerly known as non-alcoholic fatty liver disease, NAFLD).[1,2] The widespread implementation of the hepatitis B virus (HBV) vaccine has reduced acute HBV infections. However, there are still 254 million people with HBV surface antigen (HBsAg) positive chronic hepatitis B (CHB), of which 16–67% may have concurrent MASLD.[3,4] MASLD is diagnosed by the presence of hepatic steatosis (hepatocyte fat accumulation) greater than 5% without significant alcohol intake and metabolic liver dysfunction. MASLD is related to genetic susceptibility, insulin resistance, type 2 diabetes and increases the risk of central adiposity and cardiovascular disease (hypertension). Individuals with MASLD and central adiposity may have altered hepatocyte lipid accumulation, dyslipidaemia and exhibit systemic immune dysregulation.[5] Our recent study found that adult patients with obesity and MASLD have lower HBV surface antibody (anti-HBs) responses to primary HBV vaccination series, albeit protective titres (>10 mIU/mL), compared to non-obese patients.[6]

The HBV is a non-cytopathic virus, and the outcome of chronic infection is due to a dynamic interplay between the virus and host anti-viral immunity. The HBV has error-prone replication leading to evolution of viral variants under selective immune (host) pressure, which may also be impacted by MASLD and the inflammatory hepatic environment.[7] In a Chinese population, current but not past HBV (i.e. resolved) infection was associated with a decreased risk of MASLD.[8] Individuals affected by both liver diseases are at risk for accelerated liver fibrosis progression and the development of hepatocellular carcinoma (HCC).[9,10] Conversely, the presence of MASLD is also associated with reduced HBV seromarkers.[11–13] CHB patients with MASLD may have greater HBV immune control with HBV e antigen (HBeAg) loss, decreased HBV DNA levels and even HBsAg clearance (i.e. a functional cure), despite higher risk of fibrosis progression.[14] HBV suppression may be a result of enhanced immune activation and chronic hepatic inflammation which are hallmarks of MASLD.[7] Treatment with nucleos(t)ide analogue antiviral therapy (i.e. tenofovir or entecavir) can lower serum HBV DNA levels but has minimal impact on intrahepatic virus levels. It is unclear if CHB patients with low-level serum HBV DNA and MASLD would benefit from therapy to reduce liver disease and the intrahepatic viral reservoir. Recent biomarkers proposed as surrogate markers of intrahepatic HBV replication and immune responses include quantitative HBV surface antigen (qHBsAg), quantitative anti-hepatitis B core antigen (qAHBc), viral nucleic acid-related antigen (NRAg), messenger (m)RNA and pre-genomic (pg)RNA levels.[15,16] There are few studies of these novel biomarkers as well as assessment of HBV genome sequence changes in CHB/MASLD patients.

In this cross-sectional cohort study, we enrolled 53 treatment naïve CHB patients with hepatic steatosis and/or metabolic syndrome (MetS) risk factors (diabetes, dyslipidaemia and hypertension). Individuals with more hepatic steatosis showed low-level viraemia, unique HBV variants and systemic anti-viral immune responses, potentially impacting liver disease progression.

2 | MATERIALS AND METHODS

2.1 | Patient recruitment and clinical evaluation

In this cross-sectional cohort study, 53 individuals with CHB were prospectively enrolled over a 1-year period. Inclusion criteria included age of 18–60 years, CHB diagnosis (HBsAg positive >6 months), absence of end-stage liver disease (i.e. no cirrhosis or hepatocellular carcinoma) and hepatitis B treatment naïve. HBV-negative controls ($n = 12$, HBsAg negative, diagnosed with MASLD) and healthy volunteers (i.e. $n = 7$, HBsAg negative, vaccinated against HBV) were recruited for immune assay controls. All participants provided written informed consent under an approved ethics protocol (ID# REB16-0041). Whole blood was collected and processed by density gradient centrifugation to isolate plasma, serum and peripheral blood mononuclear cells (PBMCs). Demographic and clinical data included age, ethnicity (race), medical history (diabetes, hypertension, dyslipidaemia), using ethnicity specific cut-offs for obesity,[17] body mass index (BMI), waist circumference, controlled attenuation parameter (CAP) score, liver stiffness measurement (LSM) (i.e. FibroScan®), alanine aminotransaminase (ALT) and fasting glucose. Standard HBV clinical tests included HBV DNA (sensitivity 10 IU/mL), HBeAg/HBeAb and qHBsAg (Abbott Architect). Metabolic syndrome was diagnosed based on the WHO and Adult Treatment Panel (ATP) III criteria of insulin resistance and at least two diagnoses of hypertension, low high-density lipoprotein levels, plasma triglyceride >1.7 mmol/L or waist circumference (central adiposity).[18,19]

2.2 | Assessment of novel HBV biomarkers, genotyping and sequencing

Hepatitis B virus nucleic-related antigen (NRAg) and quantitative anti-HBV core (qAHBc) levels were determined using an enzyme-linked immunosorbent assay-based (Wantai Biological).[15] HBV mRNA and pgRNA levels were quantified using an established in-house method, consisting of total RNA extraction from serum using TRIzol™. The RNA extracts were digested with RNase-free DNase (Qiagen). Rapid amplification of cDNA ends (RACE) technique followed by qPCR using primers and probes complementary to the poly-A region in HBV X gene were performed to quantify HBV mRNA levels.[20] HBV pgRNA was quantified using primers and probes complementary to HBV basal core promoter. All RNA extractions and cDNA synthesis were performed with negative mock and no reverse transcriptase controls, as previously described.[21] Plasmid dilutions (concentrations from 10 to 10^7 copies/μL) were used to generate standard curves (lower limit of detection, LLOD, 50 copies/mL).

To assess HBV sequence changes, total DNA was extracted from plasma by phenol-chloroform extraction protocol and HBV DNA was amplified by direct and nested PCR using primers specific to the preC/C and preS/S regions of the HBV genome (sensitivity of 10 IU/mL or 50 virus copies/mL).[22] If needed for low-level viraemia samples, plasma was concentrated by ultracentrifugation and total HBV DNA isolated from the bottom fraction by phenol-chloroform extraction. HBV DNA-positive PCR samples were gel purified (QIAquick™ gel extraction kit, Qiagen) and analysed by Sanger sequencing (University of Calgary Sequencing Services). HBV genotype was determined using the NCBI BLAST and HBV genotyping tool. Next-generation sequencing (NGS) was done by addition of adaptor sequences to the HBV preS/S and preC/C amplicons using Phusion polymerase-based PCR (New England BioLabs) and the Illumina MiSeq platform. A PCR amplified preS/S and preC/C clone was used as an internal control to determine the NGS error rate (mean error rate calculated as <1%). All sequences were aligned using MEGA (version 10). Viral quasispecies diversity was assessed using a Kimura 2-parameter model (1000 bootstrap replicates).

2.3 | Serum cytokine quantification

A 13-plex panel of serum pro- and anti-inflammatory cytokines and chemokines were measured using multiplex Luminex assay [i.e. interleukin (IL)-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12(p70), IL-13], granulocyte monocyte colony stimulating factor (GM-CSF), interferon gamma (IFN-γ), tumour necrosis factor alpha (TNF-α) and monocyte chemoattractant protein-1 (MCP-1) (Eve Technologies). Results in CHB study subjects were compared to control cohorts (i.e. MASLD and healthy volunteers that were vaccinated against the HBV with protective immunity).

2.4 | Interferon-gamma (IFN-γ) ELISpot

To assess ex vivo HBV-specific cellular (i.e. CD4+ T helper) immunity, an IFN-γ enzyme-linked immunosorbent spot (ELISpot) assay was performed (Abcam) to recombinant HBV surface (HBcAg) or core antigen (HBcAg).[23,24] Isolated PBMCs were resuspended in RPMI 1640 with 2 mmol/L glutamine and 10% fetal bovine serum (FBS, Gibco, Thermo Fisher, Scientific). The PBMC resuspension was seeded in pre-coated mouse anti-human IFN-γ 96-well plates at a density of 10^5 cells/well. The total PBMCs were stimulated 24 h later with pre-determined optimal whole antigen concentration of 5 μg of HBsAg (adw) and 5 μg of HBcAg (American Research Products Inc.). Negative controls included unstimulated cells and dimethyl sulfoxide (DMSO) and positive control included phytohaemagglutinin (PHA) treated cells. Cells were cultured and stimulated in triplicates per condition. The stimulated total PBMCs were incubated at 37°C with 5% CO_2 for 72 h. The 72-hour incubation time was selected based on previous data comparing 24, 48, 72 vs. 96-h incubation period. The plates were washed, and biotinylated detection antibody was added as well as streptavidin-alkaline phosphatase conjugate and substrate. Formed spots were enumerated using ImmunoSpot 6 analyser (Cellular Technology Ltd.). Optimization of the ELISpot assay was done using PBMCs from a healthy individual vaccinated against HBV (Figure S1). Results were compared between subjects diagnosed with CHB- and HBV-negative patients with MASLD.

2.5 | Statistical analysis

Data were analysed in study subjects based on demographic, MASLD and metabolic syndrome clinical risk factors (i.e. diabetes, dyslipidaemia, hypertension and central obesity), and HBV biomarkers (i.e. HBV DNA, qHBsAg, HBV mRNA, HBV pgRNA, qAHBc and HBV NRAg). The body mass index (BMI) and waist circumference were used to determine obesity class/central obesity status based on ethnicity-specific cut-offs.[17] All statistical tests were non-parametric, two-sided with $\alpha = 0.05$ to account for possible differences in variance and distribution of data points. Data analysis was performed using GraphPad Prism 10.0.0 and IBM SPSS Statistics 27.0.1.0.

3 | RESULTS

3.1 | Summary of demographic, clinical and viral characteristics

In total, 53 untreated patients with CHB were prospectively recruited (Table 1). The median age was 45 y (SD = 10.6), 35% F, 56% Asian, 20% Black and 3% White. The majority were HBeAg negative (50/53, 94%) and 63% genotype B or C. The mean BMI was 28.2 kg/m^2 (SD = 4.2), 20% (11/53) had diabetes, 24% (13/53) hypertension and 35% (19/53) dyslipidaemia. The median ALT was elevated ~1.5 upper limit normal (41.9 U/L, SD = 24.0), but normal mean liver stiffness measurement (LSM) by FibroScan® of 5.7 kPa (SD 1.9) and controlled attenuation parameter (CAP) score of 306 dB/m (SD 49), indicating minimal (stage 0–1) hepatic fibrosis and moderate-to-severe hepatic steatosis (i.e. representing 67% fatty change in the liver) based on non-invasive assessment.[25,26] Most patients had low-level viral replication with mean HBV DNA and quantitative HBsAg of ~3.0 $\log_{10}$IU/mL (SD = 1.8) and HBV pgRNA 2.1 $\log_{10}$ copies/mL (SD = 1.3). Analysis of other novel exploratory viral biomarkers showed qAHBc levels of 4.7 $\log_{10}$ IU/mL (SD = 0.6) and NRAg 10.8 $\log_{10}$ IU/mL (SD = 9.1). CHB study subjects also diagnosed with MetS ($n = 17$, 32%), dyslipidaemia ($n = 19$, 35%), hypertension ($n = 13$, 24%) and/or diabetes ($n = 11$, 20%) had significantly lower levels of qHBsAg ($p = .003$ MetS, $p = .039$ dyslipidaemia, $p = .028$ hypertension and $p = .247$ diabetes) and NRAg ($p = .029$ MetS, $p = .362$ dyslipidaemia, $p = .499$ hypertension and $p = .038$ diabetes) compared to CHB study patients without these

Age (SD), years	45 (10.6)
% Female	19 (35%)
Ethnicity	30 (56%) Asian, 11 (20%) Black, 2 (3%) White, 18% (10) Other/unknown
Mean BMI (SD), kg/m^2	28.2 (4.2)
Diabetes, *N* (%)	11 (20%)
Hypertension, *N* (%)	13 (24%)
Dyslipidaemia, *N* (%)	19 (35%)
Mean ALT (SD), U/L	41.9 (24.0)
Mean LSM (SD), kPa	5.7 (1.9)
Mean CAP (SD), dB/m	306.4 (49.0)
HBeAg status, *N* (%)	50 (94%) HBeAg negative
HBV genotype	6 A (11%) 11 B (20%) 23 C (43%) 9 D (16%) 3 E (5%) 1 Unknown
Mean HBV DNA (SD), $\log_{10}$ IU/mL	3.2 (1.8)
Mean qHBsAg (SD), $\log_{10}$ pg/mL	2.9 (1.2)
Mean HBV RNA (SD), $\log_{10}$ copies/mL	1.8 (1.8)
Mean HBV pgRNA (SD), $\log_{10}$ copies/mL	2.1 (1.3)
Mean qAHBc (SD), $\log_{10}$ IU/mL	4.7 (0.6)
Mean NRAg (SD), A/CO, 450/630 nm	10.8 (9.1)

TABLE 1 Summary of demographic, virological and clinical data of 53 chronic hepatitis B (CHB) study participants.

Abbreviations: ALT, alanine aminotransaminase (normal <35 in males, <25 U/L in females); BMI, body mass index; CAP, controlled attenuation parameter; HBeAg, HBV E antigen; HBV, hepatitis B virus; LSM, liver stiffness measurement; mRNA, messenger RNA; NRAg – HBV nucleic acid-related antigen; pgRNA, pre-genomic RNA; qAHBc, quantitative anti-hepatitis B core antibody; qHBsAg, quantitative HBV surface antigen.

risk factors. CHB patients with MetS and diabetes were also found to have significantly higher HBV S gene diversity ($p = .022$ and $p = .007$, respectively; Figure 1A–D).

3.2 | Analysis of HBV sequence and viral genome diversity in patients with CHB, hepatic steatosis and/or metabolic risk factors

The HBV has error-prone replication leading to viral evolution and variants under selective conditions (i.e. anti-viral immune pressure) within the HBV S and C gene regions that are reported to be associated with clinically relevant outcomes and liver disease.[27,28] HBV sequence changes may occur due to metabolic dysregulation and inflammation associated with MASLD. Greater frequencies of HBV S gene variants associated with immune escape phenotype (i.e. P127L/T, M133L/I/T, S143L or T143S, and E164D/G) were found in CHB patients with moderate-to-severe hepatic steatosis (CAP >280 dB/m, indicating greater than 60% hepatic steatosis; $p = .046$, $p = .018$, $p = .042$, $p = .001$, respectively) compared to CHB patients with mild hepatic steatosis (CAP < 280 dB/m) (Figure 2A). Assessment of HBV C gene variants associated with HBeAg inhibition and advanced liver disease showed higher levels of mutations reported in association with cirrhosis and HCC development (i.e. E77Q, A80I/V/L and L116I) in CHB patients with CAP >280 dB/m ($p = .008$, $p = .048$, 0.049, respectively). Interestingly, more G1896A mutations associated with inhibition of HBeAg expression was also observed in CHB patients with higher hepatic steatosis (CAP >280 dB/m, $p = .008$; Figure 2B). Subjects with metabolic syndrome and more severe hepatic steatosis (CAP > 280 dB/m) also showed greater HBV S gene diversity (Figure 1A). A positive linear relationship was found between qHBsAg levels and HBV S gene diversity ($r^2 = 0.450$, $p = .017$), HBV DNA levels and HBV S or C gene diversities ($r^2 = 0.087$, $p = .327$; $r^2 = 0.073$, $p = .422$; $r^2 = 0.278$, $p = .044$; and $r^2 = 0.420$, $p = .031$), consistent with virus evolutionary dynamics (i.e. higher replication is associated with more diverse quasispecies development; Figure S2).

3.3 | Elevated systemic and Th1 cytokine responses in CHB/MASLD patients

The increased viral diversity and presence of HBV S or C gene mutations in subjects with more severe hepatic steatosis indicate enhanced systemic and/or HBV-specific immune response associated with metabolic syndrome. We investigated the non-specific

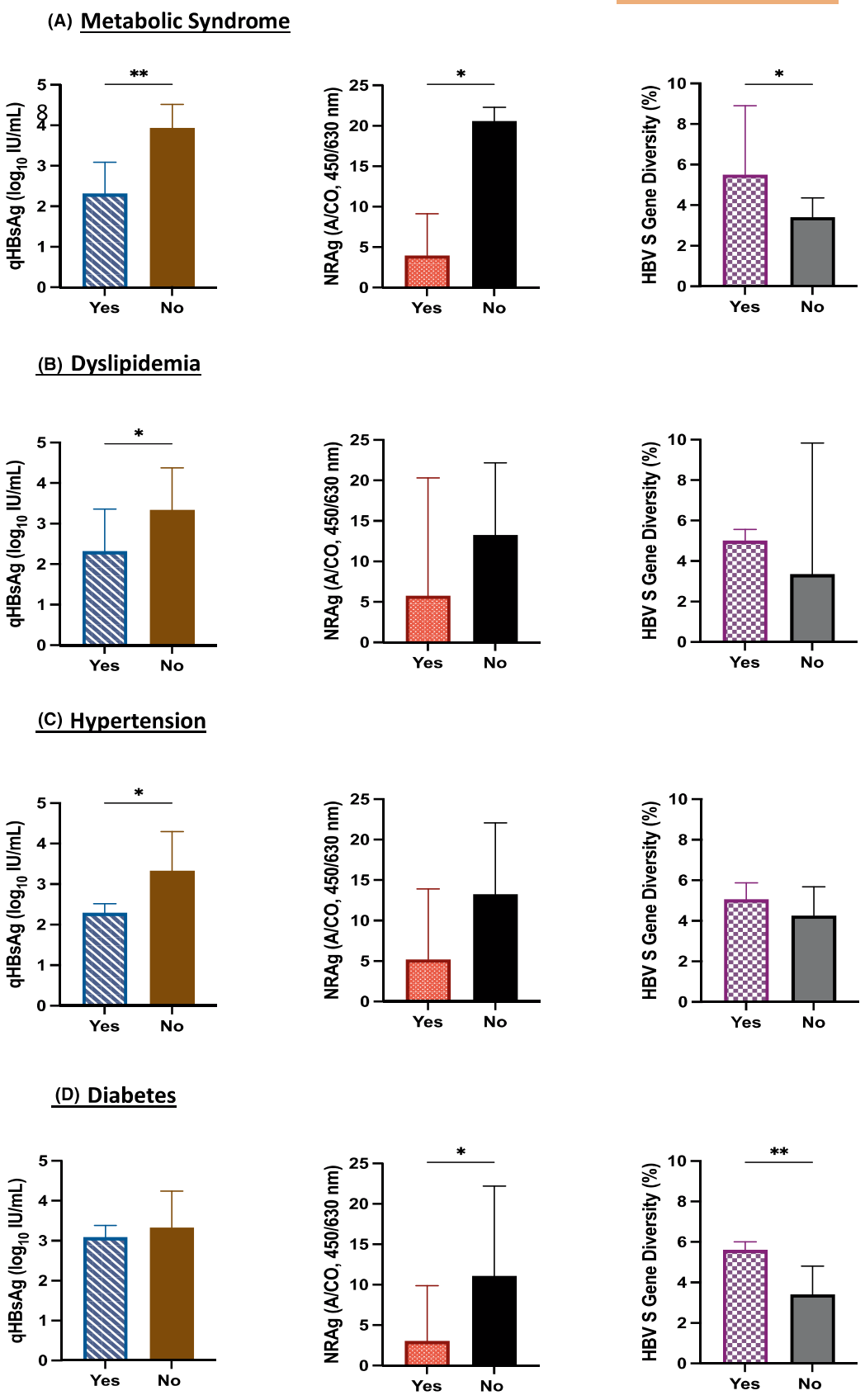

FIGURE 1 Study participants with chronic hepatitis B (CHB) and metabolic syndrome (A) or associated risk factors of dyslipidaemia (B), hypertension (C) and diabetes (D) have lower quantitative (q)HBsAg and/or nucleic acid-related antigen (NRAg) levels and increased HBV surface (S) gene diversity than those without metabolic syndrome risk factors. Mann–Whitney *U*-test was performed and median with interquartile ranges were plotted. *$p < .05$; **$p < .01$. Metabolic syndrome was classified based on the WHO and Adult Treatment Panel (ATP) III criteria of insulin resistance and at least two of the following of hypertension, low high-density lipoprotein levels, plasma triglyceride levels greater than 1.7 or central obesity based on waist circumference.[17,18]

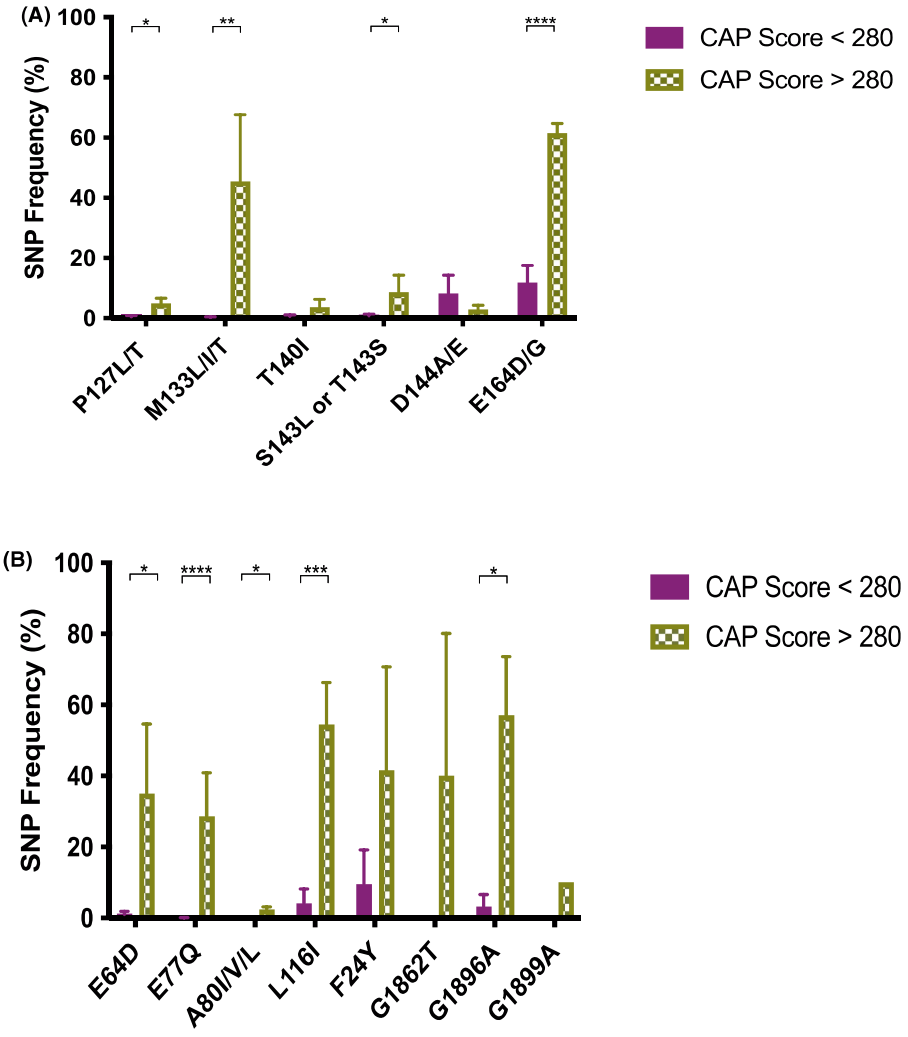

FIGURE 2 Deep sequencing analysis of HBV surface (S) and core (C) gene in 53 study participants with chronic hepatitis B (CHB). Subjects with higher hepatic steatosis (i.e. CAP score > 280 dB/m, representative of >60% hepatocyte steatosis) had increased frequencies of single nucleotide polymorphisms (SNPs) that have been reported in the literature to be associated with host–anti-viral immune escape in the HBV surface (S) gene (A), and increased risk of cirrhosis and/or development of hepatocellular carcinoma, including pre-core mutant (HBeAg-negative chronic hepatitis B) (B). Mean with standard error of the mean bars are plotted (Mann–Whitney *U*-test). CAP, controlled attenuation parameter. $p < .05$; $^{**}p < .01$; $^{***}p < .001$; $^{****}p < .0001$.

peripheral cytokine landscape by measuring a panel of 13 cytokines and chemokines and found a heightened pro-inflammatory cytokine response (including Th1 cytokines) in CHB patients with and without severe steatosis or metabolic syndrome risk factors (Figure 3). CHB patients with more severe hepatic steatosis (CAP > 280 dB/m) had higher IL-8 ($p = .0002$) and MCP-1 ($p < .05$) levels (Figure 3A); patients with diabetes had higher IL-8 ($p < .01$) and IFN-γ ($p = .011$) levels (Figure 3B); and patients with BMI > 30 and central obesity had higher IL-10 ($p < .01$) and TNF-α levels ($p = .004$) compared to CHB patients without these metabolic risk factors (Figure 3C). In comparison to patients who only had MASLD and healthy volunteers, CHB patients with mild (CAP<280) and severe steatosis (CAP >280) had increased levels of Th1 cytokine IFN-γ and/or pro-inflammatory cytokines such as IL-1β, IL-12p70 and GM-CSF (Figure 3D).

3.4 | CHB/MASLD patients have increased HBV-specific IFN-γ T cell responses

We assessed ex vivo HBV-specific total PBMC cell responses to recombinant whole HBsAg and HBcAg between CHB patients with and without severe hepatic steatosis (CAP >280 dB/m cut-off) as well as HBV-negative controls (with MASLD, mean CAP = 326 dB/m) or healthy volunteers vaccinated against HBV (i.e. received the HBsAg recombinant vaccine). HBV-specific T-cell function was analysed between cohorts and healthy vaccinated control by comparing IFN-γ secretion (i.e. CD4+ T-cell cytokine release based on number of spot forming units, SFU) and spot size. Although HBV cellular immune responses to HBsAg were found in all groups analysed (cut-off of 25 IFN- γ SFUs/10^5 PBMCs), T-cell responses were significantly higher in CHB patients with more severe hepatic steatosis (Figure 4A, $p = .021$). As expected, ex vivo HBV-specific T-cell responses to HBcAg were observed only in HBsAg-positive (CHB) patients (47%, 7/15 patients with CAP >280 dB/m vs. 40%, 6/15 patients with CAP <280 dB/m; Figure 4B).

4 | DISCUSSION

Metabolic-dysfunction associated steatotic liver disease is a multisystemic disease that is associated with a spectrum of metabolic syndrome (Mets) conditions, including dyslipidaemia,

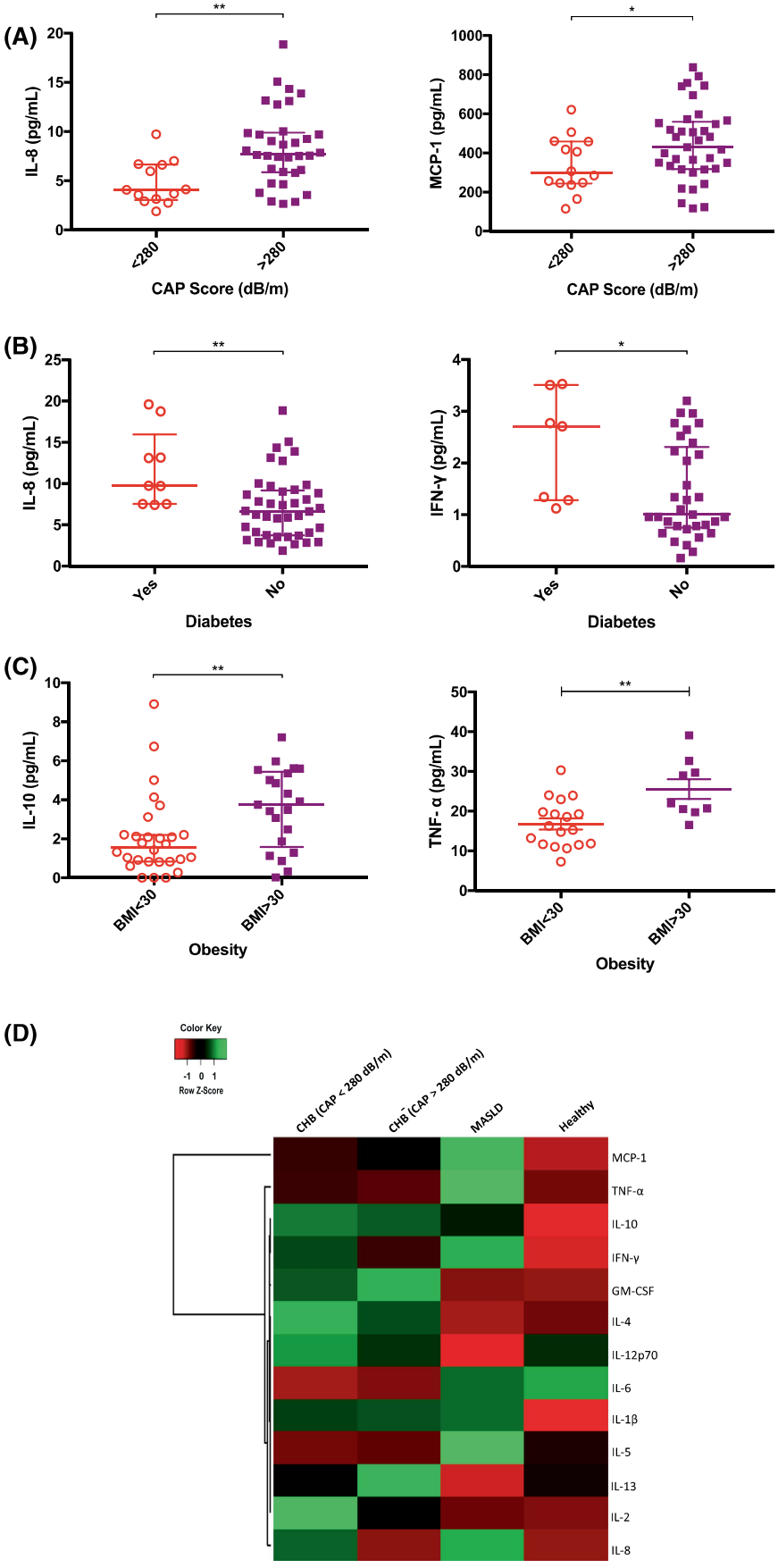

FIGURE 3 Chronic hepatitis B (CHB) patients with high CAP scores (>280 dB/m) and metabolic syndrome risk factors showed elevated serum pro-inflammatory cytokine levels. Study participants with severe steatosis had increased levels of interleukin (IL)-8 and MCP (monocyte chemoattractant protein)-1 (A); individuals with diabetes had higher levels of IL-8 and Interferon-gamma (IFN-γ) (B), and subjects with body mass index (BMI) > 30 (class 3 obesity) had higher levels of IL-10 and tumour necrosis factor-alpha (TNF-α) (C). Mann–Whitney *U*-test. Medians with interquartile ranges shown. $^{*}p < .05$; $^{**}p < .01$. (D) Clustered heat map showing comparison of all serum cytokine levels between CHB patients with low and high CAP scores, MASLD only and healthy volunteers. The scale gradient represents the Z score of normalized serum cytokine levels. Green and red colours indicate high and low expression, respectively.

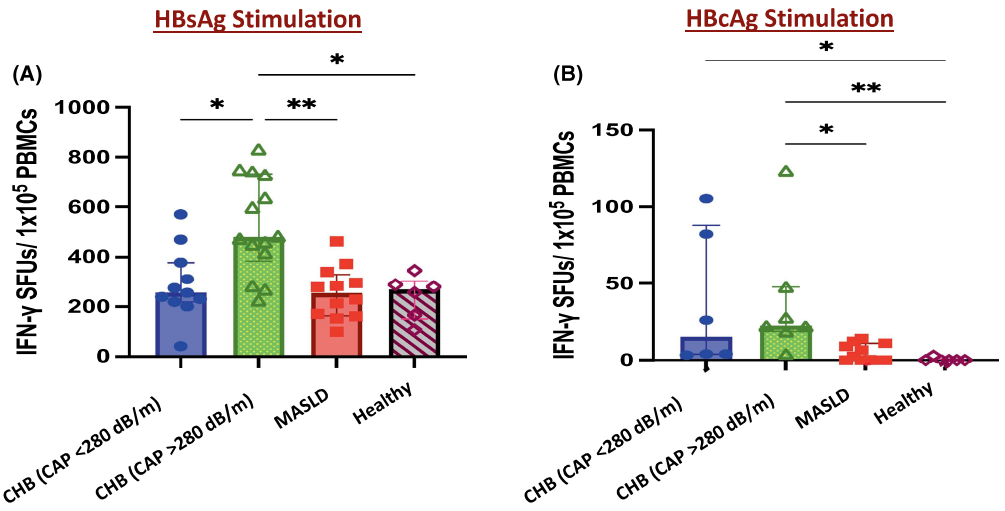

FIGURE 4 Assessment of peripheral HBV-specific T-cell frequencies in 30 chronic hepatitis B (CHB) study subjects with or without severe hepatic steatosis ($n = 15$ per group), based on controlled attenuation parameter (CAP) > 280 dB/m and diagnoses of metabolic dysfunction associated steatotic liver disease (MASLD). Interferon-gamma (IFN-γ) ELISpot assay was performed to measure and analyse HBV-specific total peripheral blood mononuclear cells (PBMC) responses to recombinant HBV surface and core whole peptides. The visualized spot forming unit (SFU) counts were normalized and a threshold of 25 IFN-γ SFU/10^5 PBMCs considered a positive response. CHB patients with CAP >280 dB/m showed more IFN-γ (i.e. CD4+ T-cell cytokine) SFUs to HBsAg (A), but no difference in response to HBcAg (B). Experiments were done in parallel with PBMC from patients with MASLD ($n = 12$, median CAP 326 dB/m (SD 55) and healthy HBV immune volunteers ($n = 7$). Kruskal–Wallis with post hoc Dunn's multiple comparisons test was performed and medians with interquartile ranges are shown.

hypertension, obesity and diabetes.[5,18,19] Large clinical cohort studies and meta-analyses indicate that MASLD may be protective against the hepatitis B virus (HBV) with suppression of HBV replication, yet worsen liver disease outcomes (cirrhosis and HCC)[9–11,13,14]. However, there is limited mechanistic understanding of the complex biological interaction between HBV within a diseased hepatocyte affected by steatosis and the inflammatory environment associated with hyperglycaemia and insulin resistance. We aim to determine the potential synergistic impact of MASLD and hepatitis B by comprehensive evaluation of virological and immunological outcomes in 53 treatment naïve CHB patients with and without severe hepatic steatosis and metabolic syndrome risk factors. We assessed HBV replication status using standard and novel viral biomarkers, HBV variants by deep sequencing of the HBV surface and core genes, serum cytokine levels and ex vivo functional HBV-specific (total PBMC) cellular immune responses to whole recombinant HBsAg and HBcAg. All study subjects had no or minimal fibrosis and low-level HBV DNA at baseline and individuals with metabolic syndrome (MetS) and associated risk factors had significantly lower levels of novel replication biomarkers (i.e. qHBsAg and NRAg levels) suggesting more robust immune response. Deep sequencing analysis showed higher HBV surface gene diversity and more frequent mutations associated with HBV surface gene immune escape (i.e. immunodominant 'a' determinant region variants) and virus core gene variants reported in the literature in association with liver disease (i.e. pre-core mutant HBeAg-negative infection). CHB study subjects with more severe hepatic steatosis showed increased peripheral systemic and Th1 cytokine levels and cellular response to whole viral proteins. Overall, the accumulated study data are consistent with other published work showing lower HBV viraemia, including novel replication biomarkers, in CHB patients with co-morbid MASLD. Moreover, our study data show an association between hepatic steatosis and HBV genome changes, systemic inflammation and host-antiviral specific immune responses, determined by assessment of HBV S gene diversity and variants, serum cytokines and IFN-gamma cellular responses to recombinant HBV proteins.

The findings from the current study contribute to mechanistic understanding of synergistic impact of MASLD and HBV infection on liver disease and CHB immunopathogenesis. Most CHB patients exhibit T-cell exhaustion (inactivation) due in part to persistent exposure to HBV antigens and a limited innate antiviral Th1 cytokine immune response (i.e. IFN-γ and IL-2) through inhibition of toll-like receptor (TLR) pathways.[29] In contrast, intrahepatic T cells and NKT cells are more prevalent in MASLD and MASH and the altered expression of saturated fatty acids (i.e. palmitate) can stimulate innate antiviral immunity (i.e. TLR4-myeloid differentiation factor 88, MyD88).[30] The MASLD hepatic immune environment is characterized by the activation of innate liver resident macrophages (Kupffer cells), and hepatic recruitment and activation of neutrophils, macrophages and natural killer cells. In humans as well as mouse models of MASLD, hepatic infiltration of T and B cells may contribute to and sustain inflammation by releasing pro-inflammatory cytokines (i.e. IFN-γ, TNF and IL-17), chemokines (i.e. MCP-1 and IL-12) and reactive oxygen species.[31] Consequently, MASLD associated immune-inflammatory profiles may enhance immune reconstitution of HBV-specific T-cell responses in CHB/MASLD patients and achieve higher rates of HBsAg loss (i.e. a functional cure), especially in patients with metabolic risk factors compared to those with only simple hepatic

steatosis. Paradoxically, the enhanced MASLD-associated inflammatory environment and HBV-specific T-cell response could exacerbate liver disease progression to fibrosis and/or HCC.

The novel molecular, virological and immunological data from the current study provide insight into the biological interactions between coexisting HBV and MASLD. However, longitudinal and large prospective clinical studies, including more diverse HBV genotypes, as well as individuals with lean MASLD/normal BMI are needed to determine causality. During the study enrolment window, most CHB patients recruited were older age, HBeAg negative and found to have elevated BMI, and few were found to have lean MASLD and/or of BMI <22.9 mm/kg^2 (normal BMI for Asian population). This is may be due to referral bias to participating clinical sites, as well as the general North American (Canadian) population who adopt a more unhealthy Western diet (high fat and refined sugar) and lifestyle. This is consistent with available data from the Canadian HBV Network retrospective registry which showed the median BMI (IQR) for 3205 patients was 24.4 (21.88–27.36 mm/kg^2) and our previous publications.[10] Furthermore, cellular immune assessment was limited to peripheral (ex vivo total PBMC responses) to recombinant whole HBV proteins in an exploratory mechanistic study. However, future work involving sorting of specific cell subsets (CD4+ T, CD8+ T cells) and stimulation with synthetic overlapping peptide pools that specifically targeting CD4+T and CD8+ T-cell epitopes are needed. Assessment of intrahepatic immune responses correlated with histopathological examination is the gold standard, but obtaining liver tissue is limited due to invasiveness, risk and patient and provider preference for non-invasive fibrosis tests. Liver stiffness measurement and CAP using FibroScan® is a validated and standardized measure of hepatic fibrosis and steatosis for patients with chronic liver disease including hepatitis B.[25,26] Liver biopsy data were only available from five CHB subjects enrolled, but histological examination was consistent with non-invasive assessment.

In summary, CHB and MASLD are both important global causes of chronic liver disease. In the current study, CHB/MASLD patients with minimal liver fibrosis and suppressed HBV replication have unique HBV surface and core gene variants and heightened peripheral (serum cytokine and ex vivo cellular) immune responses. Despite HBV suppression, co-existing MASLD leads to activation of systemic inflammatory pathways and metabolic dysregulation disrupting hepatocyte homeostasis, that may ultimately aggravate hepatitis B-related liver fibrosis progression.

AUTHOR CONTRIBUTIONS

NH Patel: Data contribution, experimental work, data analysis and manuscript draft writing and editing. A Lucko, A Vachon, C Osiowy: Data contribution, experimental work, manuscript editing and feedback. KE Doucette, A Ramji: Data contribution (patient enrolment), manuscript editing and feedback. CS Coffin: Data contribution, analysis, manuscript draft and editing, funding acquisition, support, overall responsibility for study conducts and coordination. All other authors: Data review, data analysis, manuscript editing and feedback.

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CONFLICT OF INTEREST STATEMENT

NH Patel, A Lucko, A Vachon, A Ramji, L Sycuro, TR Patel, K Chadee, G van Marle, and C Osiowy: Nothing to disclose. KE Doucette: Clinical Trials: Altimmune Pharmaceuticals, Janssen, Roche. M Raman: Consulting Fees: Takeda, Pfizer, Fresenius Kabi, Lupin. Educational Grants: Takeda, Janssen, Pfizer. CS Coffin: Advisory boards: Altimmune Pharmaceuticals, Janssen, Roche (paid to the University of Calgary, c/o the Canadian HBV Network). Consulting Fees: Gilead Sciences. Investigator Initiated Grants: Gilead, GSK, Janssen (paid to the University of Calgary c/o the Canadian HBV Network).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

This study was approved by the University of Calgary conjoint health research ethics board, CHREB (ethics ID# REB16-0041).

PATIENT CONSENT STATEMENT

All subjects provided informed written consent to participate according to the 1975 Declaration of Helsinki guidelines.

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