A Prospective Study of Hepatitis B Virus (HBV) Immunity and Hepatitis B Vaccination in Patients with Non Alcoholic Fatty Liver Disease (NAFLD) in Canada

Scientific Rationale

The Global Burden of Hepatitis B and HBV Vaccination in Canada:

The hepatitis B virus (HBV) is truly a global human pathogen that affects at least 2 billion people worldwide including ~240 million chronic hepatitis B (CHB) carriers that are at risk for end-stage liver disease [1]. The diagnosis of CHB is confirmed by the persistence of the HBV surface antigen (HBsAg) in serum for >6 months. However, a latent form of HBV infection known as occult hepatitis B infection (OBI) characterized by low-level viremia (i.e., HBV DNA < 200 IU/ml) despite undetectable serum HBsAg has been described with unclear clinical consequences [2].

A safe and effective HBV vaccine has been available for ~3 decades and consists of recombinant HBsAg which contains the major viral antigenic epitopes and induces a protective neutralizing antibody to HBsAg (anti-HBs) response in >85% of children vaccinated. Canada is a low HBV-endemic region and in Alberta, and Ontario, public health uses maternal screening for HBsAg to identify babies at-risk for CHB. Thus, all infants born to HBsAg (+) mothers are given passive-active immunoprophylaxis with hepatitis B immune globulin (HBIG) and the HBV vaccine within 12 hours of birth, as well as 2 doses at ~2 and ~6 months of age. Testing of the infants for anti-HBs is recommended at 9 months to ensure immunity. In the late 1990's, a universal HBV childhood vaccination program was initiated in all Canadian provinces and jurisdictions. In Alberta and in Ontario, school-age children are scheduled to receive the 3-dose HBV vaccine series in grade 5. However there remain a significant proportion of adult

Canadians (i.e., born before 1985) who missed childhood vaccination programs. Although current guidelines recommend that certain high-risk populations receive hepatitis B immunization (**Table 1**), appropriate identification and compliance is generally much lower in adults compared to children.

According to the most recent Canadian Association for the Study of Liver Disease guidelines [3], all adults with diabetes, as well as all patients with chronic liver disease should receive the hepatitis B vaccine. The basis for these recommendations are two-fold, (1) diabetics may be at risk of blood-borne virus (BBV) exposure through contact with contaminated blood glucose monitoring devices and (2) diabetic patients are at increased risk of the metabolic syndrome and the development of non-alcoholic fatty liver disease (NAFLD). The improvement in blood glucose monitoring devices, and increased knowledge has reduced the risk of BBV exposure in patients with diabetes. Further, our initial seroepidemiological survey of acute HBV outbreaks in Alberta revealed a decreasing prevalence in diabetic patients (**unpublished data, see Table 2**). Therefore the main incentive for HBV vaccination in diabetics is due to the concomitant risk of the metabolic syndrome and advanced liver disease due to NAFLD. There is limited data on HBV vaccination in NAFLD patients. In 1 Polish study on HBV vaccination in adults [4], good response to the HBV vaccine was observed in the patients with liver steatosis. As expected, patients with cirrhosis responded much less favorably to the hepatitis B vaccine than patients with liver steatosis. In addition, in an older study, obesity was also identified as a poor predictor of response to hepatitis B vaccination in pre-adolescents [5]. Further studies are required in a North American adult (Canadian population).

The Clinical Spectrum of Nonalcoholic Fatty Liver Disease (NAFLD) / and Non Alcoholic Steatohepatitis (NASH):

NAFLD refers to the spectrum of disease and liver damage associated with hepatic steatosis. Patients can have simple steatosis, inflammation and fibrosis, or even cirrhosis. NASH, or non-alcoholic steatohepatitis, refers to the presence of inflammation and/or fibrosis in the presence of steatosis. The prevalence of NAFLD varies widely, but in North America, it is estimated to be anywhere from 10-46%, with the prevalence of NASH ranging from 3-5% in the average population [6,7]. NAFLD, and NASH, are most commonly seen in patients who are obese, diabetic, or have other features of the metabolic syndrome (defined as the presence of 3 of the following: abdominal obesity, hypertriglyceridemia, low HDL, insulin resistance/diabetes, and hypertension).

Obesity is a growing epidemic in North America, with rates of obesity in the adult population increasing from a national average of 13% in 1962 to 34.9% in 2012 [8]. Since diabetes is closely related to obesity, rates of diabetes have also risen accordingly, with 9.3% of the US population, or 29.1 million people, having diabetes, although an estimated 27.8% of people are undiagnosed (CDC National Diabetes Statistics Report 2014).

The pathophysiology of NASH is thought to be due to a multi-hit model, although it has not been fully explained. The initial hit, or insult, is thought to be secondary to steatosis. Steatosis, which arises from triglyceride accumulation, can be due to abnormal free fatty acid metabolism (either increased import into the liver, decreased export out of the liver, or abnormal beta-oxidation) [9]. Increased delivery is often seen in settings such as obesity, excess carbohydrate and fat consumption, or rapid weight loss. Defective secretion of triglycerides is often seen in patients with gene mutations resulting in abnormal cholesterol metabolism [10]. The

second insult is thought to result in inflammation and eventually fibrosis. Some of the main mechanisms behind steatohepatitis are abnormal fatty acid β- oxidation and oxidative injury. Insulin resistance plays a role in both the development of hepatic steatosis and steatohepatitis, even in non-obese patients [11]. In the obese patient, as visceral adipose tissue increases, peripheral and hepatic insulin resistance develop and worsen, resulting in hepatic steatosis and inflammation [11,12]. Obese patients have more visceral fat, which in turn is associated with higher levels of inflammation and pro-inflammatory cytokines [13]. Obesity, older age, Hispanic origin, diabetes, and the presence of inflammation and/or fibrosis on biopsy are predictors of progression to cirrhosis [14-17]. As with other hepatic diseases, the presence of multiple hepatic processes resulting in inflammation hastens the development of fibrosis. Therefore, in patients with NAFLD, the development of acute or chronic hepatitis B infection can result in an increased risk of development of cirrhosis. Studies have shown that in pediatric patients with NAFLD, rates of non-immunity to hepatitis B are alarmingly high, approaching nearly 50% [18]. Although there have been many studies examining the prevalence of serological immunity to hepatitis B in patients with alcoholic liver disease, and the effect of concomitant infection in addition to the liver disease, no such similar studies exist examining the prevalence of hepatitis B antibodies in the adult population with NAFLD.

In summary, there is a paucity of recent studies on HBV serology and HBV vaccine responses in adults, especially in the context of obesity and NAFLD. We propose that adults with NAFLD should undergo comprehensive screening for hepatitis B immunogenicity, in addition to screening for infection, and catch up or booster vaccinations should be administered to non-immunized patients with confirmatory immunity testing thereafter.

Hypotheses/Aims:

(1) Due to missed childhood vaccination programs, the majority of adult patients with NAFLD in Canada do not have immunity to hepatitis B. (2) Adults with NAFLD who receive the HBV vaccine have reduced immunogenic responses in the setting of obesity (i.e., protective anti-HBs titres). **Aims:** (1) To determine the sero-prevalence of immunity against hepatitis B in a cohort of prospectively evaluated adult NAFLD patients. (2) To prospectively determine HBV vaccine responses (anti-HBs titres) in adult NAFLD patients.

3.0: Study Design and Methods

Study Population:

Study Population, Clinical Assessment, Inclusion/Exclusion Criteria:

Patients will be recruited from large tertiary liver clinics in Canada (Dr. C. Coffin, University of Calgary, Dr. Mang Ma University of Alberta, Dr. Curtis Cooper, University of Ottawa, Dr. Edward Tam, LAIR Centre Vancouver, and Dr. Alnoor Ramji, St. Paul's Hospital, BC) and following signed informed consent will be prospectively enrolled under an approved ethics protocol according to the declaration of Helsinki. Multi-site data will be collected using a web-based electronic report form in a secure computing environment custom developed by the PI and funded by a Canadian Foundation for Innovation infrastructure grant. Baseline data collected will include: (i) epidemiological information such as, age, sex, alcohol use, smoking; (ii) anthropometric data (body mass index, BMI, and waist circumference), (iii) liver disease lab tests such as, complete blood count, transaminases and other liver function tests, liver imaging, and non-invasive assessment of liver fibrosis via transient elastography (i.e., TE, FibroScan®), liver biopsies, and inflammatory markers associated with disease progression (ferritin, hemoglobin A1C, HbAIC, lipid profile), (iv) HBV serology including HBsAg, anti-HBs titres

and total HBV core antibody (anti-HBc) by conventional assays (iv) tests for exclusion of other liver diseases (i.e., HCV, autoimmune hepatitis, alpha 1 antitrypsin deficiency, hemochromatosis etc.) and, pregnancy. The investigators have access to large patient cohorts and are ideally positioned to perform this study. The Calgary Liver Unit services a catchment area of two million individuals and receives approximately 40 to 60 new NAFLD referrals a month, or approximately 20% of all new hepatology referrals. Inclusion criteria: Subjects 18-60 years of age, who provide signed informed consent, with a diagnosis of NAFLD/NASH according to expert assessment (by imaging, TE, abnormal lab tests and/or liver biopsy) and no evidence of prior infection or immunity to hepatitis B (negative HBsAg, anti-HBs, anti-HBc). Exclusion criteria: Subjects < 18 years of age, refused vaccination, have documented immunity / prior exposure to hepatitis B (i.e., positive for ant-HBs, anti-HBc, HBsAg), pregnancy, HIV-positive, decompensated cirrhosis (i.e., Child-Pugh Class B or C) due to impact on immune response. Subjects >60 y will be excluded, due to effect of age and reduced response to HBV vaccination. The results of a recent pooled analysis of GSK clinical trial data (N=2620, total vaccinated cohort) showed a statistically significant decrease with age in sero-protection rates, and a predictive model that showed the anti-HBs sero-protection rate remains ≥90% up to 49 y of age and ≥80% up to 60 y of age [19].

Study Design and Outcomes

Following initial clinical investigations (i.e., labwork and transient elastography as described above), patients are triaged as low, moderate, or high risk based on expert clinical consensus opinion and current clinical trial protocols in NAFLD/NASH. Low risk patients are those who do not have any risk factors for fibrosis progression, liver decompensation or HCC, or development of NAFLD complications, based on presence of dyslipidemia, diabetes, and HbAIC

levels (**Table 3**). These patients then attend a 3-hour teaching session with a dedicated NAFLD nurse clinician, a dietician, an exercise counselor, and also a social worker. Patients who have risk factors for progression to fibrosis, or who already have fibrosis are seen in clinic by a hepatologist. Finally, patients with metabolic syndrome, poorly controlled diabetes or hyperlipidemia that is difficult to treat are seen in a multidisciplinary clinic by a hepatologist and dedicated general internist with an interest in vascular risk modification. Thus, the highest risk patients are seen by multiple specialists in order to maximize care of all their medical issues, especially as they relate to NAFLD and NASH. Currently, no other clinics of this kind (either the low risk or multidisciplinary clinics) exist of their kind in Canada.

Baseline HBV Serology Testing and HBV Vaccination in Patients with NAFLD/NASH:

All subjects enrolled will be assessed for HBV serology by conventional immunoassays (i.e., HBsAg, anti-HBs, anti-HBc) to determine the prevalence of protective HBV immunity, either from natural exposure or immunization. At-risk individuals will be prospectively immunized for hepatitis B with standard dose of vaccine as per standard of care, at 0, 1 and 6 months (20 microgram intramuscular). Patients will be subsequently tested at 7 months (~1 month after completion of the vaccine series) for anti-HBs titres by conventional enzyme immunoassay (EIA, Abbott Architect). If titres are low, subjects will be given a second round of a series of 3 vaccinations, as per guidelines and re-tested for anti-HBs titres. Additionally, PBMC will be isolated from ~ 40 - 60 ml of peripheral blood according to standard protocol on Ficoll density gradient centrifugation at 2 time-points: (1) prior to first vaccine dose and (2) following the 3rd vaccine dose, at the time of serological testing for anti-HBs titres. All PBMC samples will be cyropreserved in liquid nitrogen until use, under conditions optimized in our lab

for storage and recovery of PBMC for functional studies. To assess HBV specific T and B cell responses, cyropreserved PBMCs will be thawed, re-suspended in RPMI/fetal calf serum and stimulated in quadruplicates of 3 x 10^5 cells/well with a 5 µg/ml recombinant HBsAg (American Research Products, Waltham, MA), a negative control HCV peptide pool (*kindly provided by Dr. T.I. Michalak)*, and 1 µg/ml of phytohemagglutinin (PHA; Invitrogen, Carlsbad, CA) or DMSO. HBV memory B-cell responses will be determined by an HBsAg-IgG B cell enzyme linked immunospot (ELISpot assay). IFN-gamma-secretion by HBV specific T cells will also be enumerated by ELISpot [19]. The number of specific spots (i.e., the number of spots in the presence of antigen minus the number in the absences of antigen) will be determined and a positive HBsAg specific T cell responses defined as >3-fold than the negative control (DMSO) background response and compared to the baseline (i.e., prior to vaccination) PBMC sample [20]. The PBMC cell supernatant released from unstimulated and stimulated cells will also be collected at 72 hours and cytokine secretion [21,22] (i.e., IL-2, IL-4, IL-10, TNF-alpha, IL-6, and IFN-gamma etc.) quantified using commercially available ELISA kits according to manufacturers protocol. All laboratory investigations are considered standard of care except for the extra blood draw for PBMC isolation and further testing as noted. All subjects will be monitored after each vaccine dose for possible adverse events (A/Es), as per standard clinical care. Most A/Es expected are mild (i.e., injection site redness).

4.1.3. Sample Size Calculation and Data Analysis Plan:

The primary objective of the study is to determine how NAFLD-associated metabolic risk factors and liver inflammation / fibrosis affects vaccine response. The primary outcome is anti-

HBs titres (mIU/ml), and HBsAg specific T and B cell response (i.e., IFN-gamma producing T cells and B cells by ELISpot assay, cytokine response after *ex-vivo* stimulation of PBMC), measured at 1 month after completion of the vaccine series (i.e., the recommended time for assessment of vaccine responses according to the Public Health Agency of Canada). We expect that protective immunity to the HBV vaccine will correlate with obesity risk assessment (i.e., lean-low = BMI < 34.9, medium-high = BMI >35). Subjects with anti-HBs titres ≥10 mIU/ml will be considered as responding to the vaccine while patients with anti-HBs <10 will be considered as vaccine non-responders. The literature has shown that in vaccination of 427 pre-adolescents, obesity was a significant predictor of poor immune response (P = 0.015) [23]. In another study of 239 adult health care workers, older age (> 50 y) and diabetes was a predictor of non-response [24]. A study in cirrhotic patients (N=52) showed that cirrhosis, especially alcoholic chronic liver disease, was associated with lower antibody responses compared to the general population [25]. There is limited data in adult obese patients with NAFLD and/or related fibrosis, thus a convenience pilot sample of 70 subjects stratified by lean-low and medium-high risk obseity groups, adjusting for potential confounding variables affecting vaccine response (i.e., age, sex, BMI, co-morbidities. Based on preliminary results as well as supporting animal model data (Joshi S et al., abstract presentation 2019, EASL) this sample size will be sufficient to detect a difference with Power 80% and 5% Type I error.

It is the current standard of care in our clinic to evaluate all new patients referred for hepatitis B, and to recommend HBV vaccination if non-immune. Thus, based on the number of new referrals seen (40 - 50 NAFLD patients/per month at the University of Calgary and similar numbers in other Canadian sites), we expect there will be sufficient patients recruited. Analyses will be performed using SAS 9.3 (SAS Institute, Cary NC). Summary statistics will be presented as

mean ± standard deviation for continuous data while for categorical data it will be presented as proportions. The chi-square and Fisher exact tests will be used to look at the association between immunological responses with the categorical variables. Comparisons of mean anti-HBs titres for patients with and without protective antibodies, ELISpot data and cytokine responses, within and between groups will be made using Student's t-test and analysis of variance (ANOVA) if the distribution is normal, while the Mann-Whitney U test and Kruskall-Wallis tests will be used if normality assumption is violated. The association between obesity as well as NAFLD and vaccine response will be analyzed using a logistic regression model adjusting for known factors impacting vaccine responses (i.e., age, co-morbidities).

4.1.4. Potential Limitations of HBV Vaccination Studies in NAFLD/NASH Patients: Some subjects may be lost to follow-up or refuse participation (i.e., due to religious or cultural objections to vaccination). Additionally some patients may decline to undergo a liver biopsy, which is important to confirm a histological diagnosis of NAFLD/NASH. However, it is standard practice for the PI to perform liver biopsy on all patients in which TE cannot be accurately performed (i.e., due to obesity) or if there is elevated liver stiffness suggesting at least moderate fibrosis. Additionally, the non-invasive markers (i.e., TE, ferratin, transaminases) and presence of metabolic risk factors are accepted surrogates in clinical practice and often used to base decisions on the need for a liver biopsy and eligibility for NASH/NAFLD clinical trials. Regardless, any data obtained will generate novel data on the severity of liver histology and HBV vaccine responses. Previously, the referring physician (i.e., family Dr.) was asked to immunize the patients, which may have affected compliance and follow-up. We plan to vaccinate patients in the specialist hepatology / infectious disease clinic. A clinical research study coordinator will be responsible for contacting the patients in follow up and to provide

reminders for vaccine appointments and blood work (within 1 month due to natural anti-HBs decline after vaccination). People may be more willing to be vaccinated if prescribed by a specialist since the cost is covered by health care plan. The investigators have access to large cohorts and are ideally positioned to perform this study. The Calgary Liver Unit services a catchment area of two million individuals and receives approximately 40 - 60 new NAFLD referrals a month, or ~20% of all new hepatology referrals to our group, and similar numbers are seen in other sites. This study does not include a healthy adult control arm, due to the nature of our patient population referred who usually has underlying liver disease. However, the results can be compared to data available from multiple large vaccine efficacy clinical trials in healthy adult individuals conducted by our industry partner (GlaxoSmithKline, GSK) [19].

Expertise and Support:

Dr. Coffin (MD, MSc, Assistant Professor of Medicine) is a hepatologist with a dedicated clinical and research interest in hepatitis B. Dr. Coffin holds a CIHR New Investigator Award and is appointed to the World Health Organization Expert Advisory Panel for Essential Medicines. She co-authored the Canadian Association for the Liver Disease (CASL) guidelines on Management of Chronic Hepatitis B. She has published extensively in both HBV molecular and clinical studies and has the wet-laboratory and clinic infrastructure (i.e., clinical database, bio-banking) to support her research. Dr. Jenne (PhD, Assistant Professor of Medicine). Dr. Jenne holds a Tier 1 Canada Research Chair on Intravital Imaging in Infections. He has expertise in the role of innate immunity in viral infections. Dr. Jenne has collaborations with Dr. Matteo Iannocone for HBV transgenic mice model (see letter of support). The Canadian HBV Network: (Drs. C. Cooper, MD; S. Fung, MD; M. Ma, MD; A. Ramji, MD, C. Osiowy, PhD; E. Tam, MD;

E. Yoshida MD). The Canadian HBV network is a group of clinicians and scientists dedicated to research and improved care of hepatitis B (www.CanadianHBVNetwork.ca). Our members are on the governing board of the CASL and are involved in public education on hepatitis B and NAFLD. Thus, we are well positioned to assist in these laboratory and clinical studies, and in collaboration with our industry partner facilitate Knowledge Translation to inform public health policy and promote excellence in clinical care.

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Table 1. Canadian Association for the Study of the Liver Recommendations for HBV Vaccination

Infants of HBV+ mothers (plus HBV immune globin)
Intravenous Drug Users
Multiple sexual partners
Health care workers, including laboratory personnel
Institutionalized persons
Recipients of pooled blood products (hemophiliac)
All sexual and household contacts of HBV+ persons
Travelers to endemic countries
Dialysis
Diabetics
Chronic Liver Disease

Table 2: Proportion of Hepatitis B (Acute & Chronic) cases with Diabetes in Alberta 2000-2014

Year	Alberta Proportion
2000	5.6%
2001	4.1%
2002	3.2%
2003	4.3%
2004	3.4%
2005	2.6%
2006	3.0%
2007	1.5%
2008	4.7%
2009	3.9%
2010	4.6%
2011	2.5%
2012	3.0%
2013	2.3%
2014	1.3%

Notes: Alberta Health and Wellness tracks all cases with Hepatitis B diagnosed included those with acute and chronic infection. The cases identified were merged using their provincial health care number with a provincial diabetes registry. The Diabetes registry was created using a validated National Surveillance Case definition. The diabetes and hepatitis B diagnoses were matched regardless of date, thus it is not possible to determine whether the diagnosis of diabetes was before or after the diagnosis of hepatitis B infection.

https://clinicaltrials.gov/ct2/show/NCT02466516?term=nonalcoholic+fatty+liver+disease+AND+Gilead&rank=1