

Serum viral load at the virological relapse predicts subsequent clinical flares in chronic hepatitis B patients off entecavir therapy

Short title: *clinical hepatitis following HBV relapse off ETV*

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ABSTRACT

Background: Therapeutic duration of nucleos(t)ide analogues for chronic hepatitis B (CHB) is not indefinite in many parts of the world. Viral reactivation is common off therapy but the risk of subsequent clinical outcome remains unclear and unpredictable.

Aim: We aimed to quantify the incidence of and explore the predictors for clinical flare following virological relapse in CHB patients who discontinue entecavir therapy.

Methods: This multicenter cohort study prospectively monitored 133 CHB patients who were HBeAg-negative and viral DNA-undetectable when discontinuing entecavir after at least 3 years on therapy. Following virological relapse (viral DNA >2,000 IU/mL) that occurred in 92 patients, the incidences of subsequent clinical flare and persistent (unremittent for 3 months) or severe hepatitis (with jaundice or coagulopathy) were determined, and risk factors were explored. Patients did not resume antiviral therapy until occurrence of persistent or severe hepatitis.

Results: The cumulative incidence of clinical hepatitis 2 years after virological relapse was 61.0% (95% CI, 49.9-72.3%) and that of persistent or severe hepatitis was 53.0% (95% CI, 40.9-66.2%). Serum viral load at the virological relapse was associated with both clinical hepatitis (adjusted hazard ratio [HR], 1.31 per log IU/mL; 95% CI, 1.07-1.60) and persistent or severe hepatitis (adjusted HR, 1.63 per log IU/mL; 95% CI, 1.27-2.10), after adjustment for serum aminotransferase and alfa-fetoprotein levels in the multivariate analysis. Viral

DNA >100,000 IU/mL predicted a nearly inevitable occurrence of clinical flare ($P<0.0001$).

Conclusions: A high viral load at the virological relapse predicts subsequent clinical hepatitis in CHB patients who discontinue entecavir.

Keywords: chronic hepatitis B; nucleos(t)ide analogues; hepatitis B viral DNA; off-therapy relapse; outcome research

INTRODUCTION

Nucleos(t)ide analogues (NUCs) potently inhibit replication of hepatitis B virus (HBV) and have been widely used to treat patients with chronic hepatitis B (CHB) ¹⁻³. Through sustained inhibition of the viral polymerase, long-term NUC therapy is able to ameliorate hepatitis, improve hepatic function, reverse liver fibrosis, and may reduce the risk of hepatocellular carcinoma ⁴⁻⁷. However, discontinuation of NUCs usually leads to loss of viral control ⁸⁻¹⁰. Current guidelines recommend seroclearance of hepatitis B surface antigen (HBsAg) as the indicator to discontinue NUCs ^{2,3}, because it foresees off-therapy durability ^{11,12}. However, this goal is remote in the vast majority of treated patients, requiring several decades on therapy ¹³, and practically unrealistic in most HBV-endemic countries ¹⁴. Besides, indefinite use raises safety concern of prolonged exposure to an agent that works on genetic transcription ¹⁵.

It remains controversial whether CHB patients can safely discontinue NUCs before HBsAg loss. How to predict durable off-therapy remission has become the focus of intense research¹⁶. Previous studies including ours reported that therapeutic duration, age, and serum level of HBsAg were associated with risk of relapse after NUC cessation¹⁷⁻²². We have found that HBsAg lower than 10 IU/mL at the end of therapy signifies a negligible risk of relapse, but only a small fraction of patients (less than 10%) could achieve this endpoint after 3 years of treatment²². Most patients who attempt to stop NUCs with a higher HBsAg level still carry a substantial risk of relapse.

It is not yet clear when patients should resume antiviral therapy after discontinuing NUCs. Specifically, whether retreatment is indicated for resurge of viremia or can be deferred until occurrence of clinical flare remains debatable²³. Much of the controversy arises from the paucity of data regarding clinical consequences subsequent to viral relapse. Also, little is known about the risk predictors. In this study, we aimed to clarify the incidence of clinical flares following virological relapse in CHB patients off entecavir therapy. Moreover, we hypothesized that serum levels of viral DNA or HBsAg could predict the risk.

METHODS and MATERIALS

Study design and setting

This is a prospective cohort study conducted in 3 teaching hospitals in Taiwan. Study protocol has been previously detailed ²². Briefly, we prospectively screened consecutive patients who were going to discontinue NUCs in the E-Da Hospital (Kaohsiung, Taiwan), the Lotung Poh-Ai Hospital (Yilan, Taiwan), and the National Taiwan University Hospital Yun-Lin Branch (Yunlin, Taiwan) between July 1, 2011 and January 31, 2016. Institutional review board in each hospital approved the protocol and subsequent data analysis (EMRP100-049, EMRP-104-131). Written informed consent was obtained from all patients.

Participants

Patients were eligible if they were 20 years or older, diagnosed with CHB for longer than 6 months, treated continuously with entecavir for a minimum of 3 years, and seronegative for both HBeAg and viral DNA at treatment cessation. Those with any of the following exclusion criteria were excluded: co-infection with hepatitis C or human immunodeficiency virus, cirrhosis, hepatic encephalopathy, variceal bleeding, malignant disease, organ transplantation, exposure to interferon alpha for one month or longer, and concurrent use of cytotoxic or immunosuppressive regimen. Cirrhosis was diagnosed either by liver biopsy or by clinical criteria, which included evaluation of liver surface, parenchyma, vascular structure, and splenic size.²⁴ In the absence of splenomegaly, varices

had to be shown on upper endoscopy to ascertain a clinical diagnosis of cirrhosis.

Antiviral treatment

The National Health Insurance in Taiwan has been reimbursing Taiwanese residents with CHB for antiviral therapy since October, 2003. Details of the regulations have been documented^{5,6}. The regimen was generally 3 years in duration, except for special conditions such as cirrhosis, organ transplantation, and malignancy requiring cytotoxic chemotherapy. One-year consolidation following HBeAg seroconversion was granted to pretreatment HBeAg-positive patients.¹⁻³ After expiration of the reimbursement, patients who could not pay for the regimen had to stop treatment because of the strict insurance policy.

Methods of measurement

Participants were enrolled when they discontinued NUCs, and were followed up every 3 months thereafter. At enrollment, they were interviewed for demographic data, physically checked, and examined for biochemical, serological (HBsAg, HBeAg, anti-HBs, anti-HBe), and virological tests (viral DNA). At each follow-up visit, they received physical as well as laboratory examinations.

Blood samples were collected and sent to the central laboratory in the Taipei Pathology Institutes (Taipei, Taiwan) for quantification of HBsAg and viral DNA. The former was performed using the micro-particle immunoassay (Abbott Architect i2000, Abbott Park, IL, USA). If the concentration exceeded the automatic range of the machine (0.05~250 IU/mL),

the sample was manually diluted without an upper limit. Viral DNA was quantified by polymerase chain reaction (COBAS TaqMan HBV Test, version 2.0, Roche Molecular Systems, Inc., USA) with a detection range from 20 to 1.7×10^8 IU/mL.

Definitions of outcomes after cessation of NUCs

Virological relapse was defined by serum viral DNA $>2,000$ IU/mL according to the Asian-Pacific guidelines¹. Clinical flare following virological relapse was defined by elevation of serum alanine aminotransferase (ALT) >2 folds the upper limits of normal (40 U/L). Persistent hepatitis indicated clinical hepatitis that lasted for 3 months and was defined by 2 or more measurements of serum ALT >80 U/L taken at least 3 months apart. Severe hepatitis denoted clinical flare accompanied with jaundice (serum bilirubin >2 mg/dL) or coagulopathy (prolongation of prothrombin time >3 seconds).

Patients did not resume antiviral therapy for virological relapse alone or clinical hepatitis that was self-limited. Per the regulations of the national health insurance, they would be reimbursed for retreatment if clinical hepatitis persisted for 3 months or longer (i.e. persistent hepatitis) or appeared with jaundice or coagulopathy (i.e. severe hepatitis). Observation continued until resumption of antiviral therapy, the last hospital visit, or the end of this study (April 30, 2016).

Statistical analyses

Continuous variables were summarized by the number and percentage, and categorical

ones by mean \pm standard deviation or median with interquartile range (IQR). We applied the Kaplan Meier method to estimate the cumulative incidences of outcomes, the log rank test to analyze the difference in failure time among cohorts, and the Cox proportional hazard model to identify predictors of clinical events following virological relapse. Persistent and severe hepatitis were analysed together because they both indicated resumption of antiviral therapy that terminated observation. The multivariate-adjusted analysis examined all probable predictors with a stepwise selection to keep significant variables in the final model. The hazard ratio (HR) along with 95% confidence interval (CI) was reported. Data was managed and analyzed with a commercial software (Stata, version 13.0; Stata Corp, College Station, TX, USA). All statistical analyses were two-sided with significance set at $P < 0.05$.

RESULTS

Characteristics of the study population

We screened a total of 284 consecutive patients who were about to discontinue NUCs, and identified 133 patients who discontinued entecavir with negative HBeAg and undetectable viral DNA in serum (Figure 1). During a mean off-therapy follow-up of 12.6 ± 8.2 months, 92 of them developed virological relapse and were enrolled into analysis (Supplementary figure 1). Table 1 summarized features of these study participants. The median duration on therapy was 36.6 (IQR, 36.3-36.9) months. All of the 25 pretreatment

HBeAg-positive patients consolidated the treatment for at least one year (median 21.7 months; IQR, 12.2-25.4 months) following HBeAg seroconversion.

Clinical flares following virological relapse

None of those who did not develop virological relapse experienced clinical flare. In virological relapsers, 52 patients encountered clinical hepatitis with a cumulative incidence of 61.0% (95% CI, 49.9-72.3%) at 2 years (Figure 2A). Of note, 20 of them simultaneously experienced virological and clinical relapses. The maximum of serum ALT among at-risk participants was highest immediately following virological relapse and appeared to decline over time (supplementary figure 2). Details of the serum ALT after virological relapse were given in the appendix (supplementary table 1).

Moreover, 37 patients had persistent or severe hepatitis with a cumulative incidence of 53.0% (95% CI, 40.9-66.2%) 2 years after virological relapse (Figure 2B). Among them, 4 presented with hyperbilirubinemia and 33 patients experienced unremitting clinical hepatitis for 3 months or longer. All resumed antiviral treatment and fully recovered, without anyone deteriorating to liver failure.

HBsAg clearance was observed only in a male patient who had virological relapse. He did not experience clinical relapse, before or after HBsAg loss.

Risk factors of subsequent clinical flares

Univariate analyses showed that the risk of clinical hepatitis was associated with serum

concentrations of viral DNA, ALT, and α -fetoprotein measured at the virological relapse, as well as ALT measured at treatment cessation (Table 2). The association with HBsAg was insignificant. Multivariate analysis adjusted for ALT levels affirmed that serum viral load at the virological relapse was a risk factor for clinical hepatitis (adjusted HR, 1.31 per log IU/mL; 95% CI, 1.07-1.60).

With regard to persistent or severe hepatitis, univariate analysis found that the risk was associated with serum levels of viral DNA, ALT, and α -fetoprotein at the virological relapse, as well as those of HBsAg and α -fetoprotein measured at the therapeutic end (Table 3). In the multivariate analysis, severity of viremia at the virological relapse was an independent risk factor for subsequent persistent or severe hepatitis (adjusted HR, 1.63 per log IU/mL; 95% CI, 1.27-2.10), after accounting for the effect of ALT at virological relapse and α -fetoprotein at treatment cessation.

Viral load at virological relapse stratifies risk of subsequent clinical flares

Study participants were grouped according to serum levels of viral DNA at virological relapse to illustrate how this measurement could identify patients with a high risk of clinical flares (Figure 3). The risk of clinical hepatitis (Figure 3A) and that of persistent or severe hepatitis (Figure 3B) both increased incrementally with serum viral load in a dose-response manner ($P_{trend} < 0.0001$). Virological relapse with HBV DNA $> 100,000$ IU/mL predicted a 2-year cumulative incidence of 89.7% (95% CI, 72.4-98.2%) for clinical hepatitis (Figure

3C), as compared with 51.3% in patients with the viremia <100,000 IU/mL (95% CI, 38.6-65.5%; $P<0.0001$). Similarly, whether the viral load exceeded 100,000 IU/mL significantly stratified the risk of persistent or severe hepatitis (Figure 3D; 88.0%; 95% CI, 68.7-97.9% versus 41.7%; 95% CI, 28.3-58.2%; $P<0.0001$).

DISCUSSION

This prospective study focuses on clinical consequences of viral reactivation after cessation of entecavir therapy in patients with CHB. We firstly quantified the substantial risk of clinical flare following virological relapse, revealing a 2-year cumulative incidence of 61.0% (95% CI, 49.9-72.3%) for clinical hepatitis and 53.0% (95% CI, 40.9-66.2%) for persistent or severe hepatitis. Furthermore, we found for the first time that serum HBV DNA level at the virological relapse was predictive of subsequent clinical hepatitis. A high viral load >100,000 IU/mL heralded persistent or severe hepatitis was nearly inevitable, and implicated an indication to resume treatment without undue delay. These findings will inform the management of CHB patients who attempt to stop NUCs before HBsAg clearance.

What off-therapy event indicates retreatment has been hotly debated. Many experts endorse viral rebound with HBV DNA greater than 2,000 IU/mL as the indication⁸, because viremia above this level is linked to excessive risk of liver cirrhosis and cancer^{25, 26}. Besides,

early retreatment abolishes untoward immune reactions elicited by viral replication and decreases the chance of severe exacerbation. However, virological relapse is so common that it almost implies NUCs should not be discontinued at all, since more than 80% of patients will need to restart treatment^{8, 17-19}. Furthermore, treating clinically silent viremia without biochemical hepatitis may hamper the opportunity of host immunity to control the virus²³. Virological relapse alone, therefore, is arguably not recommended to indicate treatment resumption¹⁶.

The major concern of stopping NUC in CHB patients is the risk of acute on chronic liver failure that may rapidly ensue after a severe bout of clinical flare. The importance of vigilant monitoring and timely retreatment cannot be overemphasized. Given the absence of liver failure in this cohort, our study suggests a follow-up interval of 3 months may suffice to avoid this dreadful complication. On the other hand, our data also demonstrated that viral replication could abruptly rebound and immediately manifest with severe clinical illness. In fact, the incidence rate of clinical flare and the severity of serum ALT elevation were all highest right after virological relapse. Therefore, it is imperative to restart treatment in those at high risk of clinical deterioration.

Based on our findings, retreatment should not be deferred in patients who relapse with a high viral load. Identification of this indicator helps to diminish the risk of persistent or severe clinical hepatitis. The predictive value of viral DNA for this scenario, which

resembles similar utility in treatment-naïve patients²⁷⁻²⁹, can be explained by the pathogenesis of liver injury resulting from the host-virus interaction. Unlike our prior work that showcased the value of HBsAg measured at the end of therapy in predicting both clinical and virological relapses²², the present research found this biomarker was no longer an independent predictor for subsequent clinical events once virological relapse has occurred. Taken together, these findings point out complementary roles of viral DNA and HBsAg in guiding the management of CHB patients off NUCs. In addition, we also found serum levels of ALT and alfa-fetoprotein at the treatment cessation were associated with risks of clinical flare and persistent/severe hepatitis, respectively. Severity of residual liver injury at the end of therapy probably underlines this association, insomuch as these two biomarkers both are indicators of hepatocyte damage.^{30,31}

The strict retreatment policy in Taiwan offers a unique opportunity to investigate the natural course of viral reactivation after discontinuation of NUCs. Only when the clinical hepatitis persisted for 3 months or manifested with hepatic decompensation would patients resume treatment. Furthermore, prospective selection, enrollment, and observation of consecutive patients in 3 recruiting sites minimized selection bias. The standardized protocol as well as measurement of viral DNA and HBsAg in a central laboratory controlled confounding from heterogeneity among different hospitals.

We nonetheless acknowledge the following limitations. First, our data could not address

long-term outcomes such as liver cirrhosis or HCC. Tackling this issue requires a huge study sample and prolonged follow-up, given the low incidence of these late complications.

Second, while we clearly showed the predictive value of viral load for subsequent clinical hepatitis, we did not intend to advocate interruption of antiviral therapy was preferred over continuous treatment. Our novel findings should be cautiously interpreted in the context of healthcare systems. Third, because this study prospectively enrolled patients at treatment cessation, we could not analyze several pretreatment factors including viral genotype and length of consolidation on therapy. It should also be noted that the study participants stopped treatment because of the insurance policy, instead of testing the APASL guidelines.³² Nevertheless, previous studies have shown a substantial risk of relapse despite fulfilling the APASL recommendation.^{8,9} Lastly, it is beyond the scope of the present study to address HBsAg loss, given that few participants lost HBsAg off therapy.

In conclusion, our study adds to current understanding about the risk and risk stratification of clinical hepatitis subsequent to virological relapse in CHB patients who stop taking entecavir. Virological relapses was followed by persistent or severe hepatitis. Moreover, this risk is predictable by serum levels of HBV DNA at the episode of virological rebound. For patients whose viremia recurs at a gradient greater than 100,000 IU/mL, the risk is so high that retreatment should not be deferred. These novel findings may help to guide CHB management off NUC therapy and warrant independent validation and further exploration.

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Accepted Article

Table 1. Characteristics of CHB patients who developed virological relapse after discontinuing entecavir therapy

Characteristics	All (<i>n</i> =92)
Male sex, <i>n</i> (%)	71 (77.2%)
Status at treatment start	
HBV DNA, log IU/ml	6.8 (4.3-8.4)
ALT, IU/mL	158 (93-451)
α -fetoprotein, ng/mL	4.5 (3.3-8.2)
Positive anti-HBe, <i>n</i> (%)	67 (72.8%)
Positive HBeAg, <i>n</i> (%)	25 (27.2%)
Status at treatment cessation	
HBsAg, log IU/mL	2.9 (2.5-3.1)
ALT, IU/mL	22 (16-35.5)
α -fetoprotein, ng/mL	2.6 (2.1-3.4)
Positive anti-HBe, <i>n</i> (%)	87 (94.6%)
Treatment duration, months	36.6 (36.3-36.9)
Status at virological relapse	
Age, years	50.2 (42.4-58.1)
HBV DNA, log IU/ml	4.1 (3.6-5.0)
HBsAg, log IU/mL	2.9 (2.5-3.3)
ALT, IU/mL	39 (22-67.5)
α -fetoprotein, ng/mL	2.5 (2.0-3.3)

Notes. ALT, Alanine transaminase; Anti-HBe, hepatitis B e antibody; CHB, chronic hepatitis B; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; NUC, nucleos(t)ide analogue; VR, virological relapse

Table 2. Cox proportional hazard model for clinical hepatitis after virological relapse

Variables	<u>Unadjusted analysis</u>			<u>Multivariate modelling</u>		
	HR	95 % CI	<i>P</i>	HR	95% CI	<i>P</i>
<u>Status at treatment start</u>						
Male sex	1.40	0.70-2.82	0.34			
Age, per year	1.007	0.98-1.03	0.55			
HBeAg positivity	0.94	0.51-1.72	0.83			
Anti-HBe positivity	1.13	0.61-2.09	0.71			
HBV DNA, per log IU/mL	1.05	0.89-1.23	0.57			
ALT, per U/L	1.0	0.999-1.0	0.32			
α -fetoprotein, per ng/mL	1.0	1.0-1.002	0.13			
<u>Status at treatment cessation</u>						
Age, per year	1.008	0.98-1.03	0.51			
Anti-HBe positivity	1.06	0.33-3.43	0.92			
HBsAg, per log IU/mL	1.50	0.94-2.40	0.09			
ALT, per U/L	1.01	1.004-1.016	0.001	1.008	1.002-1.014	0.005
α -fetoprotein, per ng/mL	1.05	0.98-1.13	0.14			
Regimen length, per month	1.02	0.99-1.06	0.23			
<u>Status at virological relapse</u>						
Age, per year	1.01	0.98-1.03	0.53			
HBsAg, per log IU/mL	1.39	0.93-2.06	0.11			
HBV DNA, per log IU/mL	1.38	1.15-1.66	0.001	1.31	1.07-1.60	0.008
ALT, per U/L	1.003	1.001-1.004	<0.001	1.002	1.001-1.004	0.002
α -fetoprotein, per ng/mL	1.14	1.05-1.25	0.003			

Table 3. Cox proportional hazard modelling for persistent/severe hepatitis following virological relapse

Variables	<u>Unadjusted analysis</u>			<u>Multivariate modelling</u>		
	HR	95 % CI	<i>P</i>	HR	95% CI	<i>P</i>
<u>Status at treatment start</u>						
Male sex	1.26	0.58-2.77	0.56			
Age, per year	1.001	0.98-1.03	0.96			
HBeAg positivity	1.44	0.75-2.79	0.28			
Anti-HBe positivity	0.73	0.38-1.43	0.36			
HBV DNA, per log IU/mL	1.16	0.96-1.41	0.12			
ALT, per U/L	1.0	0.999-1.001	0.65			
α -fetoprotein, per ng/mL	1.0	0.999-1.001	0.71			
<u>Status at treatment cessation</u>						
Age, per year	1.001	0.98-1.03	0.91			
Anti-HBe positivity	0.60	0.18-1.95	0.39			
HBsAg, per log IU/mL	1.79	1.01-3.18	0.05			
ALT, per U/L	1.003	0.995-1.012	0.43			
α -fetoprotein, per ng/mL	1.10	1.03-1.2	0.01	1.09	1.006-1.175	0.034
Regimen length, per month	1.04	0.99-1.08	0.11			
<u>Status at virological relapse</u>						
Age, per year	1.001	0.98-1.03	0.94			
HBsAg, per log IU/mL	1.56	0.96-2.53	0.07			
HBV DNA, per log IU/mL	1.63	1.33-1.99	<0.001	1.63	1.27-2.10	<0.001
ALT, per U/L	1.006	1.003-1.008	<0.001	1.005	1.002-1.008	0.001
α -fetoprotein, per ng/mL	1.16	1.05-1.28	0.003			

FIGURE LEGENDS

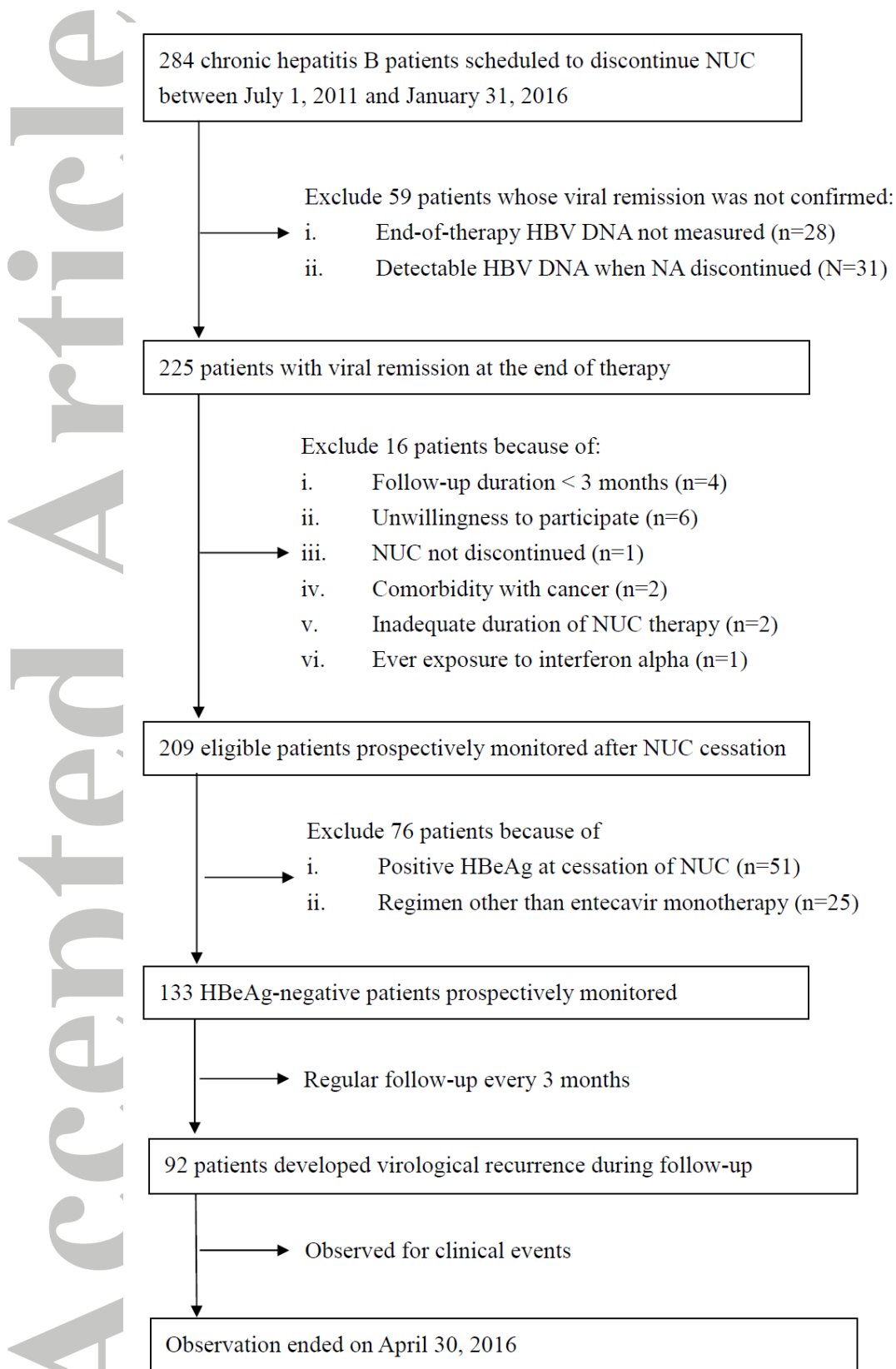


Figure 1. Study flowchart

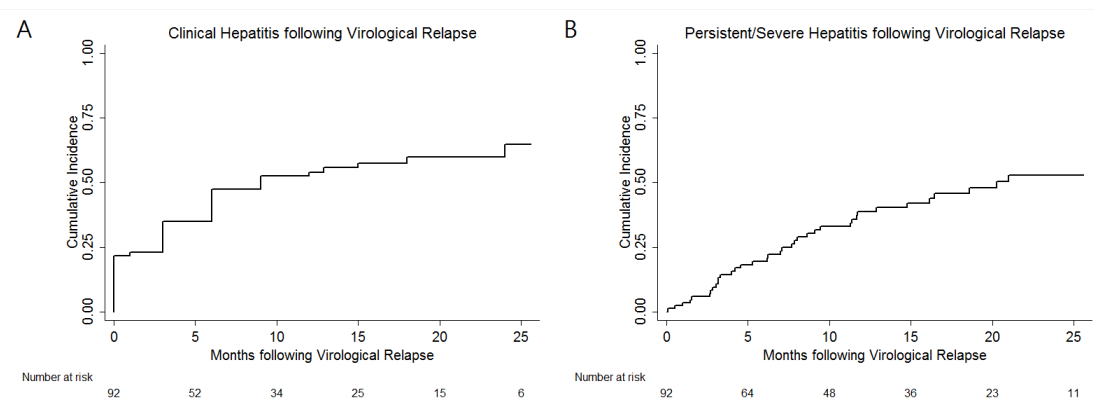


Figure 2. Cumulative incidences of clinical flares following the episode of virological relapse: clinical hepatitis (panel A), persistent or severe hepatitis (panel B)

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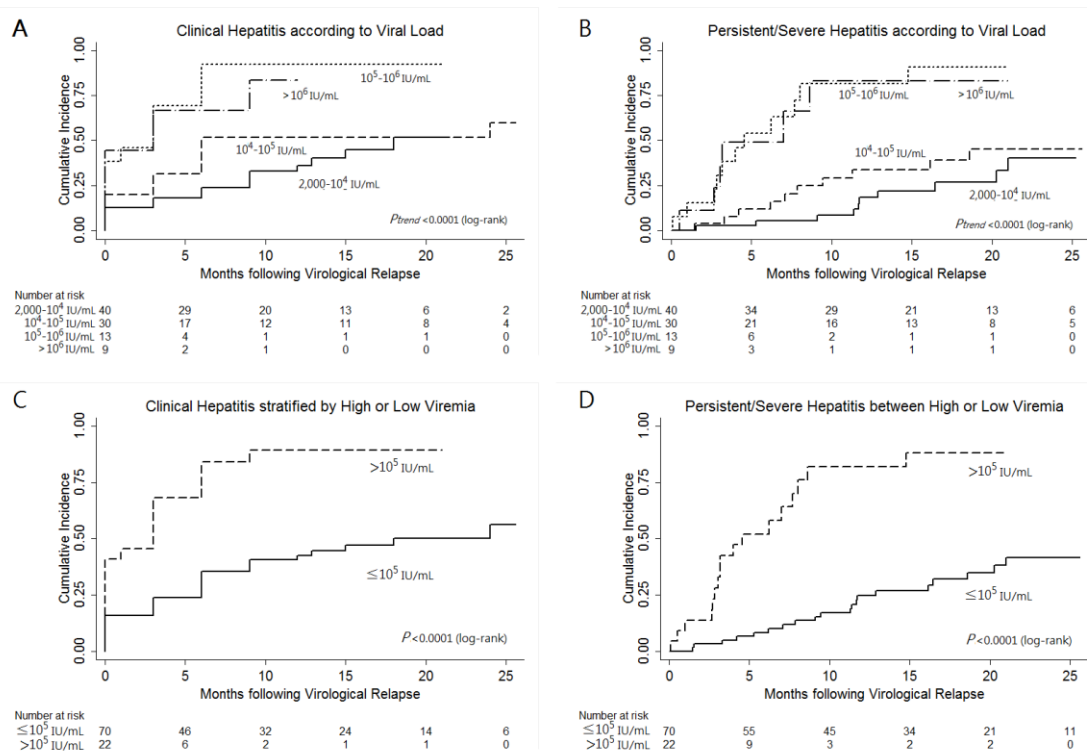


Figure 3. Serum viral load at the virological relapse stratified the risk of subsequent clinical flares: cumulative incidence of clinical hepatitis (panel A) and that of persist or severe hepatitis (panel B) according to different levels of viremia; almost all patients who relapsed with HBV DNA $>100,000$ IU/mL would subsequently develop clinical hepatitis (panel C) as well as persistent or severe hepatitis (panel D).