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# Early HBcrAg and Anti-HBc Levels Identify Patients at High Risk for Severe Flares After Nucleos(t)ide Analogue Cessation—A Pooled Analysis of Two Clinical Trials

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## ABSTRACT

**Background:** Severe flares ( $ALT \geq 10 \times ULN$ ) are a well-recognised adverse outcome after nucleos(t)ide analogue (NA) cessation and may lead to liver failure. Thus, identification of patients at risk for these flares is of major importance.

**Methods:** Data were used from two prospective studies on NA cessation conducted in the Netherlands and Canada. Patients were eligible based on EASL criteria. HBcrAg and anti-HBc levels were measured at end of treatment (EOT) and week 6 (FUW6). Logistic regression was used to study the association with severe flares.

**Results:** Seventy-eight patients were analysed with a mean age of 49 years, 16 (21%) Caucasian and a majority (65%) were treated with Tenofovir. Overall, 22 patients (28%) developed a severe flare, and 29 (37%) patients were retreated. At EOT, higher HBcrAg levels (aOR: 1.97,  $p = 0.05$ ;  $\geq 4$ log: 47% severe flare vs.  $< 3$ log: 19%,  $p = 0.036$ ), lower anti-HBc (aOR: 0.29,  $p = 0.036$ ;  $< 2$ log: 50% vs.  $\geq 3$ log: 11%,  $p = 0.029$ ) and higher HBcrAg/anti-HBc-ratio (aOR: 3.17,  $p = 0.015$ ;  $\geq 2$ : 58% vs.  $< 1.5$ : 14%,  $p < 0.001$ ) were associated with an increased risk of severe flares, adjusted for HBsAg. At FUW6, higher HBcrAg (aOR: 2.91,  $p = 0.035$ ;  $\geq 5$ log: 83%,  $< 3$ log: 4%,  $p < 0.001$ ), lower anti-HBc (aOR: 0.46,  $p = 0.29$ ;  $< 2$ log: 50% vs.  $\geq 3$ log: 0%,  $p = 0.003$ ) and higher HBcrAg/anti-HBc-ratio (aOR: 2.19,  $p = 0.048$ ;  $\geq 1.75$ : 52% vs.  $< 1.75$ : 8%,  $p < 0.001$ ) were associated with an increased risk of severe flares, adjusted for HBV DNA and ALT.

**Conclusion:** Higher HBcrAg, lower anti-HBc and higher HBcrAg/anti-HBc ratio at EOT and during the first weeks of post-treatment follow-up are associated with an increased risk of hepatic flares after NA withdrawal and could therefore potentially be used to select patients eligible for therapy cessation and to identify patients requiring retreatment.

**Trial Registration:** This study was a post hoc and follow-up study of two previously registered clinical trials (NCT01911156 & NTR7001). No new patients were prospectively included

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## 1 | Introduction

Cessation of nucleos(t)ide analogue's (NAs) is a novel strategy to increase the probability of functional cure in a selected group of chronic hepatitis B (CHB) patients, especially those with lower HBsAg-levels [1–4]. However, stopping therapy is not without risks since withdrawal could result in viral rebound potentially leading to severe hepatic flares and liver failure [5–7]. The risk of hepatic flares and the probability of achieving functional cure represent a delicate balance between host- and virus-related factors [8, 9]; a response of the immune system is required for viral clearance, whereas failure to clear the virus or hyper-activation of the immune response could lead to severe hepatic flares. Thus, identification of biomarkers that reflect this interplay is needed.

Two candidate biomarkers are hepatitis B core-related antigen (HBcrAg) and anti-hepatitis B core antibodies (anti-HBc). HBcrAg is a combination of hepatitis B core antigen (HBcAg), hepatitis B envelop antigen (HBeAg) and 22kDa precore protein (p22Cr), and serum HBcrAg correlates strongly with intra-hepatic replicational activity, even in patients who are virally suppressed with NA [10–13]. Lower HBcrAg levels at end of treatment (EOT) have previously been linked to superior virological outcomes after NA withdrawal, such as lower risks of clinical relapses and higher rates of HBsAg loss [14, 15]. However, data regarding off-treatment HBcrAg kinetics and the risk of subsequent flares is lacking.

Where HBcrAg reflects viral activity, anti-HBc may be reflective of certain aspects of the host anti-viral immune response. Anti-HBc is produced by HBV-specific B-cells as a response to Hepatitis B Core antigen and therefore reflects immune activity, with higher levels previously linked to superior immune responses [16–19]. Based on these prior observations, we hypothesised that serum levels of HBcrAg and anti-HBc, and their ratio could be used to predict the risk of severe flares after NA withdrawal.

Therefore, the aim of this study was to investigate the association between EOT and (early) off-treatment HBcrAg and anti-HBc levels and (severe) hepatic flares after treatment withdrawal.

## 2 | Methods

### 2.1 | Study Cohort

The study included patients with CHB who withdrew NA therapy during two previously published prospective studies conducted in Canada (NCT01911156) [20] and the Netherlands (NTR7001) [21]. The follow-up time after NA cessation was 72 or 96 weeks, respectively. More comprehensive details regarding the study designs have been published before [20, 21]. Patients were eligible if they were HBeAg negative at NA cessation and achieved long-term viral suppression. Exclusion criteria were a co-infection (i.e., HCV/HDV/HIV), history of advanced fibrosis or hepatocellular carcinoma (HCC) prior to NA cessation. All patients gave written informed consent for re-use of their data and samples when included in the

original studies. Both studies were approved by the research ethical board of each participating center and performed in concordance with Good Clinical Practice guidelines and the Declaration of Helsinki in 1964 as modified by the 59th World Medical Association General Assembly, Seoul, South Korea, in October 2008.

### 2.2 | Primary Outcome

The primary outcome was the association between EOT and early off-treatment HBcrAg- and anti-HBc levels and the occurrence of severe ALT-flares ( $ALT \geq 10 \times ULN$ ) during subsequent follow-up.

### 2.3 | Laboratory Assays

Quantitative HBcrAg was determined using the Lumipulse G HBcrAg assay (Fujirebio Europe, Ghent, Belgium) on the LUMIPULSE G1200 analyser (Fujirebio Inc., Tokyo, Japan), having a lower limit of detection (LOD) and quantification (LOQ) of 2 and 3 IU/mL log, respectively. Anti-HBc was quantified using ChemiLuminescent Enzyme Immunoassay (CLEIA) kits (Fujirebio Inc., Tokyo, Japan), with a LOQ of 0.1 log IU/mL. More details about the qualitative/quantitative HBsAg, HBeAg and HBV DNA assays can be found elsewhere [20, 21].

### 2.4 | Statistical Analyses

HBcrAg and anti-HBc were analysed separately after  $\log_{10}$  transformation and as a ratio (HBcrAg/anti-HBc ratio). Continuous variables were presented as mean  $\pm$  standard deviation or median [P25–P75], dependent on normal distribution. For statistical comparison, Student's t-test, Wilcoxon rank-sum- or signed-rank test (continuous data) or Chi-square test and Fisher's exact test (categorical data) were used, where appropriate. For the association between EOT levels with subsequent severe flares, a logistic regression model was constructed with the occurrence of severe flares ( $ALT \geq 10 \times ULN$ ) during the study period (76/96 weeks) as a binary outcome. Subsequently, the association between off-treatment HBcrAg and anti-HBc kinetics was studied using the 'geom\_smooth' function from 'ggplot2' package, including the 'loess' model for fitting a trend line and standard error (se) confidence interval.

In addition, the area under the receiver operating characteristic curve (AUROC) was calculated for the different biomarkers by using "pROC" package, including the occurrence of severe flares during the study period as a binary outcome.

Finally, a sub-analysis was performed using HBcrAg and anti-HBc data obtained at follow-up week 6–8 (FUW6), excluding patients who developed any flare ( $ALT \geq 5 \times ULN$ ) or were retreated before/at this visit. To avoid overfitting, logistic regression models were constructed with only the variables of interest (HBcrAg and anti-HBc), HBV DNA- and/or ALT levels.

All statistical tests were performed in SPSS statistical software [22] and R version 4.2.2 [23]. All tests were two-sided, and a  $p$ -value  $< 0.05$  was considered to be statistically significant.

### 3 | Results

In total, 78 patients discontinued NA therapy. The mean age at EOT was 49 ( $\pm 9.7$ ) years, 64% were male and 21% were Caucasian, Table 1. Mean HBsAg, HBcrAg and anti-HBc levels at EOT were 2.88 ( $\pm 0.75$ ), 3.36 ( $\pm 0.83$ ) and 2.38 ( $\pm 0.50$ )  $\log_{10}$  IU/mL, respectively. Thirty-two (41%) patients had HBcrAg  $< 3$   $\log_{10}$  IU/mL at EOT. During follow-up, 22 (28%) patients developed a severe flare, with a median time of 12 [9–19] weeks after NA cessation. Time until a severe flare was shorter in patients who discontinued TDF/TAF therapy vs. ETV (11 [9–13] vs. 27 [24–34] weeks,  $p = 0.003$ ). No patients developed hepatic decompensation or HBsAg loss afterwards. Further characteristics of these flares and outcomes have been described in the respective papers [20, 21].

#### 3.1 | End of Treatment Factors Associated With Severe Flares

Patients who developed severe flares after NA withdrawal, compared to those without, had significantly higher HBcrAg levels at EOT (3.68 ( $\pm 0.83$ ) vs. 3.23 ( $\pm 0.80$ ),  $p = 0.040$ ), whereas anti-HBc levels were lower (2.16 ( $\pm 0.54$ ) vs. 2.46 ( $\pm 0.47$ ),  $p = 0.034$ ). This was reflected in a significantly higher HBcrAg/anti-HBc ratio (1.84 ( $\pm 0.63$ ) vs. 1.39 ( $\pm 0.58$ ),  $p = 0.010$ ). No statistical differences in type of therapy (TDF/TAF: 82% vs. 64%,  $p = 0.131$ ) or duration of NA therapy (9.3 vs. 8.1 years,  $p = 0.260$ ) were found between patients with or without a severe flare. When comparing ETV with TDF/TAF-treated patients, mean HBcrAg (3.44 ( $\pm 0.93$ ) vs. 3.32 ( $\pm 0.79$ ),  $p = 0.605$ ), anti-HBc (2.34 ( $\pm 0.54$ ) vs. 2.40 ( $\pm 0.49$ ),  $p = 0.652$ ) and HBcrAg/anti-HBc ratio (1.60 ( $\pm 0.77$ ) vs. 1.48 ( $\pm 0.54$ ),  $p = 0.500$ ) were comparable at EOT.

In both univariable- and multivariable logistic regression models adjusting for HBsAg-levels at EOT, higher HBcrAg levels (aOR 1.97; 95%CI 1.00–3.90,  $p = 0.050$ ), lower anti-HBc levels (aOR 0.29; 95%CI 0.09–0.92,  $p = 0.036$ ) and higher HBcrAg/anti-HBc ratio (aOR: 3.17; 95%CI 1.25–8.07,  $p = 0.015$ ) at EOT were associated with an increased flare risk (Table 2).

The highest risk of severe flares was observed in patients with HBcrAg  $> 4$ log (47% vs. 21%,  $p = 0.026$ ), in patients with anti-HBc  $< 2$ log (50% vs. 22%,  $p = 0.032$ ) and in those with a HBcrAg/anti-HBc ratio  $\geq 1.75$  (52% vs. 17%,  $p = 0.002$ ), Figure 1. These findings were consistent in both univariable- and multivariable regression models, adjusted for HBsAg levels at EOT (Table 2).

The highest discriminatory performance was obtained with a combination of HBcrAg and anti-HBc (as a ratio), with an AUROC of 0.73, which was higher than for HBcrAg alone (AUROC: 0.68), anti-HBc alone (AUROC: 0.64) or HBsAg (AUROC: 0.56; Table S1).

#### 3.2 | Off-Treatment Biomarker Kinetics in Patients With and Without Severe Flares

As shown in Figure 2, HBV DNA increases were seen in both patients with and without flares, although peak HBV DNA levels were higher in those with severe flares (mean peak HBV DNA: 6.83 ( $\pm 0.95$ ) vs. 4.36 ( $\pm 1.57$ ),  $p < 0.001$ ). Conversely, HBcrAg remained relatively stable after withdrawal in those without severe flares, whereas an increase was seen in those who developed a severe flare (mean peak HBcrAg levels: 6.28 ( $\pm 1.44$ ) vs. 4.29 ( $\pm 1.49$ ),  $p < 0.001$ ). No statistically significant differences were seen between both groups with regards to mean peak anti-HBc (3.52 ( $\pm 0.26$ ) vs. 3.43 ( $\pm 0.29$ ),  $p = 0.23$ ) or lowest anti-HBc levels (2.12 ( $\pm 0.49$ ) vs. 2.32 ( $\pm 0.47$ ),  $p = 0.11$ ). Compared to patients who stopped ETV, patients who withdrew TDF/TAF therapy had significantly higher HBcrAg (3.90 ( $\pm 1.23$ ) vs. 3.37 ( $\pm 0.83$ ),  $p = 0.042$ ), HBV DNA (4.12 ( $\pm 1.44$ ) vs. 1.40 ( $\pm 0.56$ ),  $p < 0.001$ ), anti-HBc (2.84 ( $\pm 0.58$ ) vs. 2.31 ( $\pm 0.62$ ),  $p = 0.002$ ) and ALT-levels (1.02 [0.55–1.88] vs. 0.58 [0.47–0.78]  $\times$  ULN,  $p = 0.004$ ) at 6 weeks after NA withdrawal.

#### 3.3 | Association Between HBcrAg and Anti-HBc Levels at Off-treatment Week 6 With Subsequent Severe Flares

This sub-analysis was performed in 63 patients after exclusion of 15 patients (11 had developed a flare before this time, 1 patient was retreated and 3 patients had no sample available for measuring both markers). Patients who developed a severe flare after FUW6 had higher mean HBcrAg levels (4.71 ( $\pm 1.10$ ) vs. 3.43 ( $\pm 0.93$ )  $\log$  IU/mL,  $p < 0.001$ ), lower anti-HBc levels (2.23 ( $\pm 0.48$ ) vs. 2.69 ( $\pm 0.60$ ),  $\log$  IU/mL,  $p = 0.006$ ) and a higher HBcrAg/anti-HBc ratio (2.24 ( $\pm 0.79$ ) vs. 1.43 ( $\pm 0.89$ ),  $p = 0.003$ ) at FUW6.

In both univariable- and multivariable logistic regression models, adjusted for ALT- and HBV DNA levels at FUW6, again higher HBcrAg levels (aOR 2.91; 95%CI 1.08–7.88,  $p = 0.035$ ), lower anti-HBc (aOR 0.46; 95%CI 0.11–1.92,  $p = 0.29$ ) and higher HBcrAg/anti-HBc ratio (aOR 2.19; 95%CI 1.01–4.78,  $p = 0.048$ ) were associated with a higher risk of severe flares (Table 1,3).

The highest rate of severe flares was observed in those with HBcrAg  $\geq 5$ log (83% vs. 16%,  $p = 0.002$ ), in patients with anti-HBc  $< 2$ log (50% vs. 17%,  $p = 0.021$ ) and in those with a HBcrAg/anti-HBc ratio  $\geq 1.75$  (52% vs. 8%,  $p < 0.001$ ), Figure 3. These findings were consistent in both univariable- and multivariable regression models, adjusted for ALT- and HBV DNA levels at the same visit (Table 3).

The highest discriminatory performance was obtained with a combination of HBcrAg and anti-HBc; the AUC was 0.82 which was higher than for HBcrAg alone (AUC: 0.80), anti-HBc alone (AUC: 0.73), HBV DNA (AUC: 0.67) or HBsAg (AUC: 0.61), Table S1.

### 4 | Discussion

In this combined analysis of two prospective studies, including 78 CHB patients who withdrew NA therapy, we show

**TABLE 1** | Patient characteristics of included patients and comparison between patients with and without a severe flare ( $\geq 10 \times \text{ULN}$ ).

	Total number of patients (N=78)	No severe flare ( $\geq 10 \times \text{ULN}$ ) (N=56)	Severe flare ( $\geq 10 \times \text{ULN}$ ) (N=22)	<i>p</i> <sup>b</sup>
Patient characteristics				
Sex, male (n, %)	50 (64)	33 (59)	17 (77)	0.129
Age, years (mean $\pm$ SD)	49 ( $\pm 9.7$ )	50 ( $\pm 10.1$ )	47 ( $\pm 8.5$ )	0.257
Ethnicity, Asian/Caucasian/Other (n, %)	54 (69)/16 (21)/8 (10)	36 (64)/13 (23)/7 (13)	18 (82)/3 (14)/1 (5)	0.304
Type of therapy, ETV/TDF/TAF (n, %)	24 (31)/51 (65)/3 (4)	20 (36)/34 (61)/2 (4)	4 (18)/17 (77)/1 (5)	0.320
Duration of NA therapy, years (mean $\pm$ SD)	8.4 (4.1)	8.1 (3.9)	9.3 (4.5)	0.260
HBeAg positive at SOT (n, %)	21 (27)	11 (20)	10 (46)	0.021
Liver stiffness, kPa (mean $\pm$ SD)	4.8 ( $\pm 1.1$ )	4.8 ( $\pm 1.1$ )	4.8 ( $\pm 1.1$ )	0.738
Serology at NA cessation <sup>a</sup>				
HBsAg, log <sub>10</sub> IU/mL (mean $\pm$ SD)	2.88 ( $\pm 0.75$ )	2.83 ( $\pm 0.81$ )	3.01 ( $\pm 0.57$ )	0.273
HBsAg categorised (n, %)				0.529
> 1000	38 (49)	26 (46)	12 (57)	
100–1000	30 (39)	23 (41)	7 (33)	
< 100	9 (12)	7 (13)	2 (10)	
HBeAg, log <sub>10</sub> IU/mL (mean $\pm$ SD)	3.36 ( $\pm 0.83$ )	3.23 ( $\pm 0.80$ )	3.68 ( $\pm 0.83$ )	0.040
HBeAg, < 3 log <sub>10</sub> IU/mL (n, %)	32 (41)	26 (47)	6 (29)	0.140
Anti-HBc, log <sub>10</sub> IU/mL (mean $\pm$ SD)	2.38 ( $\pm 0.50$ )	2.46 ( $\pm 0.47$ )	2.16 ( $\pm 0.54$ )	0.034
HBeAg/antiHBc-ratio (mean $\pm$ SD)	1.51 ( $\pm 0.62$ )	1.39 ( $\pm 0.58$ )	1.84 ( $\pm 0.63$ )	0.010
ALT $\times$ ULN (mean $\pm$ SD)	0.62 ( $\pm 0.24$ )	0.59 ( $\pm 0.24$ )	0.70 ( $\pm 0.22$ )	0.061

Abbreviations: Anti-HBc, hepatitis B core antibody; ETV, entecavir; HBcrAg, hepatitis B core-related antigen; HBsAg, hepatitis B surface antigen; IQB, interquartile range; NA, nucleos(t)ide analogue; TAF, tenofovir alafenamide; TDF, tenofovir disoproxil fumarate; ULN, upper limit of normal.

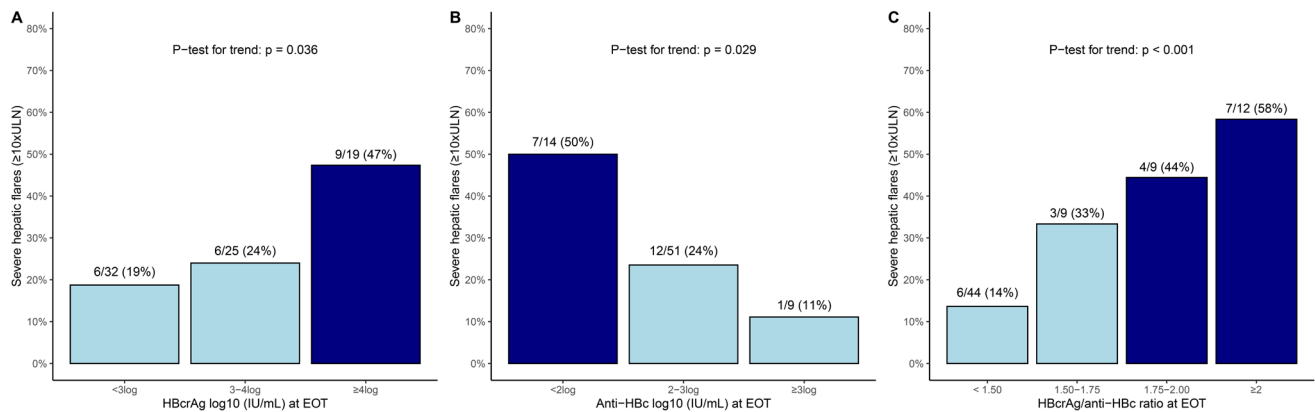
<sup>a</sup>Due to lack of sample residue, HBcrAg and anti-HBc could not be determined at end of therapy in 2 and 4 patients, respectively. HBsAg at EOT was not available from 1 patient.

<sup>b</sup>Compared using the independent t-test (normal distribution, presented as mean (SD)), Wilcoxon rank sum test (if not normally distributed, presented as median [IQR]) and Chi-square test (categorical).

**TABLE 2** | Uni- and multivariate logistic regression models for HBcrAg- and anti-HBc at end of therapy and severe flares ( $\geq 10 \times \text{ULN}$ ).

At end of therapy	Univariate models			Multivariate models <sup>a</sup>		
	aOR	95% CI	p	aOR	95% CI	p
Model 1 (continuous)						
HBcrAg ( $\log_{10}$ )	1.946	1.036–3.654	0.038	1.974	0.999–3.901	0.050
Anti-HBc ( $\log_{10}$ )	0.291	0.098–0.867	0.027	0.294	0.094–0.921	0.036
Model 2 (categorical)						
HBcrAg ( $\log_{10}$ ) $\geq 4.00$	3.375	1.120–10.169	0.031	3.032	0.953–9.645	0.060
Anti-HBc ( $\log_{10}$ ) $\geq 2.00$	0.277	0.082–0.932	0.038	0.321	0.090–1.141	0.079
Model 3 (continuous)						
HBcrAg/anti-HBc ratio	3.167	1.259–7.964	0.014	3.174	1.248–8.068	0.015
Model 4 (categorical)						
HBcrAg/anti-HBc ratio $\geq 1.75$	5.378	1.760–16.431	0.003	5.846	1.852–18.454	0.003

Abbreviations: Anti-HBc, anti-hepatitis B core; aOR, adjusted odds ratio; HBcrAg, hepatitis B core related antigen; HBsAg, hepatitis B surface antigen.  
<sup>a</sup>Adjusted for HBsAg ( $\log_{10}$ ) levels at end of therapy.



**FIGURE 1** | Rates of severe flares ( $\geq 10 \times \text{ULN}$ ) during 72–96 weeks follow up based on HBcrAg and anti-HBc levels at end of therapy. Dark blue bars = sub-group with the highest risk of severe flares, light blue bars = sub-group with lower risk of severe flares.

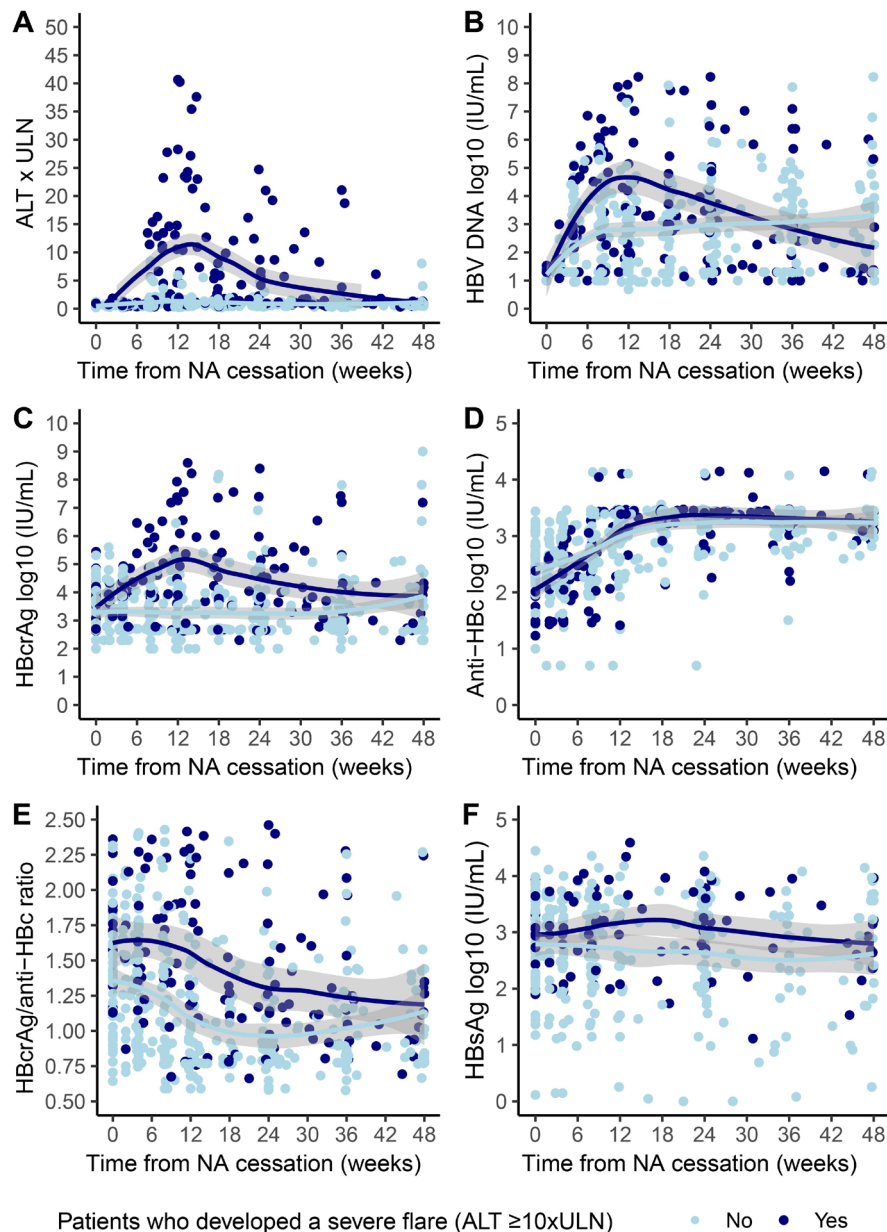
that higher HBcrAg, lower anti-HBc and a higher HBcrAg/anti-HBc ratio at EOT are associated with a higher risk of severe off-treatment flares. Furthermore, higher HBcrAg, lower anti-HBc and a higher HBcrAg/anti-HBc ratio at FUW6 were also highly predictive of subsequent flares, independent from HBV DNA. HBcrAg and anti-HBc levels, and their ratio, can therefore potentially be used to guide follow-up and the decision to restart antiviral therapy in patients who discontinue NA therapy.

Withdrawal of NA therapy has recently emerged as a novel strategy to increase functional cure rates. Unfortunately, these potential benefits are counterbalanced by a significant risk of severe viral rebound and subsequent hepatic flares, which may precipitate liver failure and death [5–7]. In the current cohort, severe flares were observed in 28% of patients, which is rather high compared to previously described [24]. A possible explanation could be the restrictive retreatment criteria that were applied in both studies. This emphasises

that risk stratification is essential for optimal patient selection for finite therapy and to identify patients who require urgent retreatment.

Although previous studies [15, 25–29] have suggested that EOT HBcrAg and anti-HBc could be predictive of virological outcomes after NA withdrawal, the current report describes the first study on the use of combining HBcrAg and anti-HBc levels for assessing the risk of severe flares after NA cessation. This was enabled by the prospective design with restrictive retreatment criteria, which resulted in high rates of severe flares. In our cohort, patients with higher HBcrAg and lower anti-HBc levels were at increased risk of severe flares after therapy withdrawal. Prohibitively high flare rates were observed for patients with HBcrAg levels  $\geq 4\log$  (47%), anti-HBc levels  $< 2\log$  (50%) and high HBcrAg/anti-HBc ratio ( $\geq 1.75$ : 52%) at EOT. Conversely, patients with low HBcrAg and/or high anti-HBc were at reduced risk of flares, and these patients may therefore be the best candidates for NA withdrawal. Findings





**FIGURE 2** | Kinetics after NA cessation using a trend line including standard error for (A) ALT×ULN, (B) log<sub>10</sub> HBV DNA, (C) log<sub>10</sub> HBcrAg, (D) log<sub>10</sub> anti-HBc, (E) HBcrAg/anti-HBc ratio, (F) log<sub>10</sub> HBsAg. Dark blue line = patients who developed a severe flare (≥10×ULN), light blue line = patients who didn't develop a severe flare (≥10×ULN).

were consistent in multivariable analysis, underscoring that both HBcrAg and anti-HBc, and their combination, provide important new information over existing biomarkers including HBsAg.

Since even patients with the most favourable EOT profile remain at risk for flares, careful off-treatment monitoring remains essential. Recent studies have shown that HBV DNA and HBsAg levels at off-treatment week 24 are associated with excellent long-term outcomes [30]. However, since many severe flares occur early after NA cessation (median time of 12 weeks in the current cohort), biomarkers obtained sooner after therapy withdrawal (preferable during the first weeks after withdrawal of treatment) are needed for early risk stratification [24]. Since HBsAg levels remain relatively stable (early) after therapy withdrawal, even in patients developing severe flares, the use

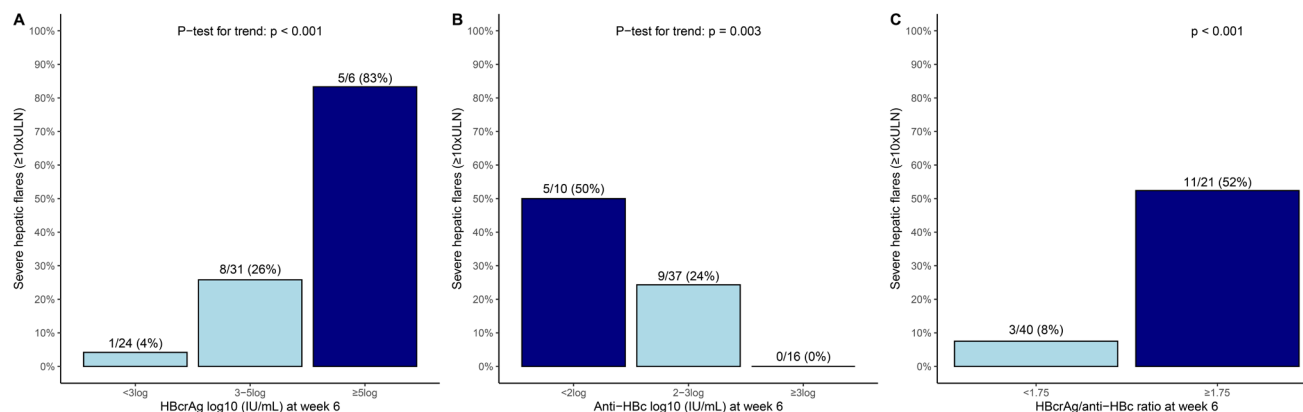
of HBsAg as a predictor during the early off-treatment phase is limited. Thus, alternative biomarkers are therefore needed to guide off-treatment follow-up and management. A recent study showed that lower HBcrAg levels after therapy withdrawal might be associated with a lower risk of viral relapse [31]. The current study now shows for the first time that HBcrAg and anti-HBc levels obtained at 6 weeks post-treatment are predictive of subsequent flares. In our cohort, both high HBcrAg and low anti-HBc and a higher ratio were associated with a higher subsequent risk of severe flares, independent of HBV DNA and ALT. For instance, severe flares were observed in nearly all patients with HBcrAg ≥ 5 log at week 6, whereas flare risk was negligible in patients with HBcrAg < 3log (4%), anti-HBc ≥ 3log (0%) and/or HBcrAg/anti-HBc ratio < 1.75 (8%). Also, it is important to note that these associations remained significant even after adjusting for HBV DNA levels at the same visit. Recently, it has

**TABLE 3** | Uni- and multivariate logistic regression models for HBcrAg- and anti-HBc at week 6 and severe flares ( $\geq 10 \times \text{ULN}$ ).

At week 6	Univariate models			Multivariate models <sup>a</sup>		
	aOR	95% CI	p	aOR	95% CI	p
Model 1 (continuous)						
HBcrAg ( $\log_{10}$ )	3.421	1.633–7.170	0.001	2.912	1.076–7.884	0.035
Anti-HBc ( $\log_{10}$ )	0.253	0.081–0.794	0.018	0.458	0.109–1.915	0.285
Model 2 (categorical)						
HBcrAg ( $\log_{10}$ ) $\geq 5.00$	25.556	2.659–245.570	0.005	16.502	1.362–199.90	0.028
Anti-HBc ( $\log_{10}$ ) $\geq 2.00$	0.205	0.049–0.856	0.030	0.152	0.027–0.857	0.033
Model 4 (continuous)						
HBcrAg/anti-HBc ratio	2.663	1.164–6.093	0.020	2.194	1.008–4.777	0.048
Model 5 (categorical)						
HBcrAg/anti-HBc ratio $\geq 1.75$	13.567	3.166–58.139	<0.001	10.815	2.340–49.998	0.002

Abbreviations: aOR, adjusted odds ratio; HBcrAg, hepatitis B core-related antigen; anti-HBc, anti-hepatitis B core.

<sup>a</sup>Adjusted for ALT  $\times$  ULN ( $\log_{10}$ ) and HBV DNA ( $\log_{10}$ ) levels at week 6.



**FIGURE 3** | Rates of severe flares ( $\geq 10 \times \text{ULN}$ ) during 72–96 weeks of follow-up based on HBcrAg and anti-HBc levels at week 6. Dark blue bars = sub-group with the highest risk of severe flares, light blue bars = sub-group with lower risk of severe flares.

been recommended to restart treatment when HBV DNA levels exceed 100,000 IU/mL [24]. Four of the 10 patients in our cohort who had HBV DNA levels  $> 5 \log$  and available HBcrAg levels at the week 6 visit developed a severe flare afterwards, of whom all had HBcrAg  $> 5 \log$ , resulting in a PPV of 80%, NPV of 100%, specificity of 83% and sensitivity of 100%. These findings suggest that HBcrAg and anti-HBc levels at week 6 could potentially be used as an additional tool to stratify flare risk, and to identify patients with such a high risk of flares that increased monitoring and consideration of restarting treatment is required. It remains, however, important to highlight that severe flares can still occur in patients with the most favourable characteristics, underscoring that careful monitoring remains essential in all patients who discontinue treatment.

Furthermore, the combination of HBcrAg and anti-HBc yielded superior accuracy over either marker alone. This might be due to the fact that these biomarkers combine two aspects that determine the outcome of NA withdrawal: viral replication and

the host immune response. Our study, therefore, introduces new clinical applications for these markers. Interestingly, thresholds predictive of a low risk of flares are well above the lower limits of quantification for these markers, overcoming a limitation typically observed in studies aiming to predict HBsAg loss [32, 33].

Our study also sheds some new light on the previous observation that withdrawal of TDF therapy is a risk factor for hepatic flares [24, 34]. Flares after TDF cessation occur earlier and are more severe compared to flares occurring after ETV withdrawal [24, 35, 36]. Despite similar HBcrAg and anti-HBc levels at EOT, (early) HBcrAg- and anti-HBc kinetics seemed to differ between patients who withdrew TDF/TAF vs. ETV, with both higher HBcrAg and higher anti-HBc levels observed at 6–8 weeks after withdrawal of tenofovir therapy.

The current study has several strengths, including the prospective designs of the included cohorts with detailed off-treatment follow-up data available, and the multiethnic

population enrolled. The main limitation of our study is the limited sample size and thus validation of our findings in a larger cohort is needed. Furthermore, the cohort included both pretreatment HBeAg-positive and pretreatment HBeAg-negative patients. Since pretreatment HBeAg-positive patients may have higher HBcrAg levels, we also performed stratified analyses and found consistent results, although statistical significance could not be obtained due to lack of statistical power (Figure S1).

In conclusion, higher HBcrAg, lower anti-HBc and a higher HBcrAg/anti-HBc ratio at end of therapy and at FUW6 seem to be associated with a higher risk of severe flares after therapy withdrawal. If confirmed, these markers could potentially be used to identify patients with such a high risk of flares that therapy withdrawal should be discouraged and/or early retreatment advised. Nevertheless, a risk of flares remains even in patients with favourable characteristics, thus necessitating careful follow-up in all patients who discontinue treatment.

#### Author Contributions

**Edo J. Dongelmans:** writing – original draft, methodology, visualization, writing – review and editing, conceptualization, investigation, formal analysis, data curation. **Jordan J. Feld:** data curation, writing – review and editing. **André Boonstra:** writing – review and editing, data curation. **Sylvia M. Brakenhoff:** writing – review and editing, data curation. **David Wong:** writing – review and editing, data curation. **Colina Yim:** writing – review and editing, data curation. **Mark Claassen:** writing – review and editing, data curation. **Pieter Honkoop:** writing – review and editing, data curation. **Bettina E. Hansen:** writing – review and editing, data curation. **Robert A. de Man:** data curation, writing – review and editing. **Scott Fung:** data curation, writing – review and editing. **Thomas Berg:** data curation, writing – review and editing. **Florian van Bömmel:** data curation, visualization. **Harry L. A. Janssen:** data curation, writing – review and editing, conceptualization, funding acquisition, investigation, methodology, supervision. **Milan J. Sonneveld:** data curation, supervision, methodology, writing – review and editing, conceptualization, investigation, funding acquisition.

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#### Ethics Statement

This study was approved by the research ethical board of each participating centre.

#### Conflicts of Interest

J.F. receives research grants from Abbvie, Gilead, Janssen, Enanta, Eiger and is a consultant for Abbvie, Gilead, Finch, Arbutus and GlaxoSmithKline. A.B. has been in consulting or in advisory boards for Gilead Sciences and Bristol-Myers Squibb and has received research grants from Roche, Gilead Sciences, Fujirebio and Janssen. B.E.H. has received grants from Intercept, CymaBay, Albireo, Mirum, Calliditas and Gliad and is a consultant for Intercept, CymaBay, Albireo, Mirum,

Genfit, Calliditas, Eiger, and ChemomAb. H.L.A.J. received grants from: Gilead Sciences, GlaxoSmithKline, Janssen, Roche, Vir Biotechnology Inc. and is consultant for: Aligos, Gilead Sciences, GlaxoSmithKline, Janssen, Roche, Vir Biotechnology Inc., Precision Biosciences and Griffols. M.J.S. receives speakers' fees and research support from Roche, Bristol Myers Squibb, Gilead Sciences and Fujirebio. All other authors disclose no conflicts.

#### Data Availability Statement

The data that support these findings are not publicly available, since they are subject to (inter)national data protection laws to ensure data privacy of the study participants. The data can therefore not be shared.

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### Supporting Information

Additional supporting information can be found online in the Supporting Information section.