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BRIEF REPORT

HBcrAg and cirB-RNA Do Not Predict Clinical and Virological Outcomes in Patients With HBeAg-Negative Chronic Infection

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Keywords: cirB-RNA | functional cure | HBcrAg | HBeAg-negative chronic infection | HBsAg | novel biomarkers

ABSTRACT

Background & Aims: Predicting clinical and virological outcomes in HBeAg-negative (HBeAg-neg) chronic infection often requires long-term monitoring. Our study explored whether a single measurement of quantitative HBsAg (qHBsAg), HBV core-related antigen (HBcrAg), and circulating HBV RNA (cirB-RNA) can define the natural course of untreated HBeAg-neg chronic infection patients.

Methods: To this aim, we included 128 naïve HBeAg-neg chronic infection patients, stratified according to qHBsAg levels in: (1) 10–1000 IU/mL, (2) 1000–10 000 IU/mL, and (3) > 10 000 IU/mL.

Results: HBcrAg and cirB-RNA were detected in 27% and 19% of patients with qHBsAg > 1000 IU/mL but rarely detected in patients with qHBsAg < 1000 IU/mL. After a median follow-up of 5.1 years, 9.4% of patients lost HBsAg, and 8.5% experienced an increase in HBV DNA > 2000 IU/mL. qHBsAg < 1000 IU/mL was the only factor independently associated with functional cure.

Conclusions: In untreated HBeAg-neg chronic infection patients, single-point cirB-RNA and HBcrAg do not offer additional predictive value over qHBsAg < 1000 IU/mL for spontaneous HBsAg loss.

Abbreviations: ALT, serum alanine aminotransferase; cccDNA, covalently closed circular DNA; cirB-RNA, circulating HBV RNA; HBcrAg, hepatitis B core-related antigen; HBeAg, hepatitis B e antigen; HBeAg-neg, HBeAg-negative; HBV, hepatitis B virus; IQR, interquartile range; LLoQ, lower limit of quantification; NAs, nucleos(t)ide analogues; pegIFNα, peginterferon-alfa; qHBsAg, quantitative HBsAg.

Sara Battistella and Thais Leonel contributed equally to this work.

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Summary

- A single measurment of cirB-RNA and HBcrAg does not predict outcomes in naive HBeAg-negative chronic infection patients.
- Quantitative HBsAg levels below 1000 IU/mL predict functional cure and identify patients with minimal risk of disease progression.
- In patients with low quantitative HBsAg, extending follow-up intervals may be a safe cost-effective strategy.

1 | Introduction

The natural history of hepatitis B virus (HBV) infection is classified into different phases based on hepatitis B e antigen (HBeAg) status, HBV DNA levels, and serum alanine aminotransferase (ALT) [1]. HBeAg-negative (HBeAg-neg) chronic infection is considered a benign condition characterised by low viral load, persistently normal ALT, and minimal or no hepatic necroinflammatory activity and fibrosis [1]. However, these patients may experience fluctuating HBV DNA levels. Therefore, long-term observation and frequent assessments are recommended to accurately classify them within the HBeAg-neg chronic infection phase. Furthermore, the probability of achieving a functional cure and the timing of such an outcome can vary. Several biomarkers have been proposed to better classify these patients, guide their clinical management, and avoid unnecessary follow-up. While quantitative HBsAg (qHBsAg) has been proposed as a useful tool to distinguish HBeAg-neg inactive from active disease [2], its potential value as a serological biomarker to define different sub-populations within the HBeAg-neg chronic infection phase—such as those who progress to chronic hepatitis or the "grey zone", those who remain stable over time, and those who achieve a functional cure—remains to be determined. Recently, new biomarkers such as hepatitis B core-related antigen (HBcrAg) and circulating HBV RNA (cirB-RNA) have been proposed as surrogate markers of covalently closed circular DNA (cccDNA) transcriptional activity [3]. Undetectable HBcrAg (lower limit of quantification [LLoQ] < 3 logU/mL) has been shown to be a useful marker to differentiate HBeAg-negative chronic infection from chronic hepatitis [4]. Similarly, the detection of cirB-RNA has been shown to be a valuable marker to differentiate various phases of HBV infection, as well as to predict the response to peginterferon-alfa (pegIFNα) treatment and nucleos(t)ide analogues (NAs) off-treatment relapse in selected cohorts of patients [5, 6]. Nevertheless, most studies have not used standardised assays to quantify cirB-RNA, hindering data reproducibility. An automated cirB-RNA assay (for investigational use) capable of detecting viral RNAs expressed from cccDNA, with a LLoQ of 10 copies/mL, might enhance the assessment of this marker in HBeAg-neg patients [7–9]. The combination of HBcrAg and cirB-RNA has been proposed to better characterise the natural history of chronically infected HBV patients and guide antiviral treatment withdrawal [10]. Nevertheless, most studies have focused on patients with chronic hepatitis and/or those on treatment with NAs [5, 11]; thus, their role in assessing the outcomes of naïve HBeAg-neg chronic infection patients still needs clarification [10]. In addition, although HBcrAg and cirB-RNA detectability is predominant among patients with either HBeAg-positive or negative active hepatitis, these markers are also occasionally positive in patients with chronic infection, although the significance of this finding is not well established [4–6].

Therefore, our study aimed to characterise HBeAg-neg chronic infection patients according to qHBsAg levels and the presence or absence of HBcrAg and cirB-RNA at a single time point, to investigate how these biomarkers can help predict clinical and viral outcomes during follow-up, including HBsAg loss.

2 | Methods

2.1 | Patients

We included all HBeAg-neg chronic infection patients followed at the Liver Unit of the Hospital Clinic, Barcelona. Patients were classified as HBeAg-neg chronic infection according to EASL Guidelines 2017 [1]. None of the patients was under antiviral therapy when the samples were obtained. All patients were HBeAg-negative with positive anti-HBe, had HBV DNA levels persistently below 2000 IU/mL and normal ALT levels for at least 1 year. Patients were classified into 3 groups according to their baseline qHBsAg levels: group (1) qHBsAg between 10 and 1000 IU/mL ($n = 56$); group (2) qHBsAg between 1000 and 10 000 IU/mL ($n = 35$); and group (3) qHBsAg > 10 000 IU/mL ($n = 37$). Demographical and clinical variables were collected at baseline. In addition, patients were followed every 6–12 months with liver tests.

This study was approved by the Ethical Committee of Hospital Clinic and all participants provide their informed consent.

2.2 | Viral Biomarkers

HBV DNA was determined by real-time PCR using the Cobas HBV test on the Cobas 6800 automated system Roche Diagnostics, Mannheim, Germany; LLoQ < 10 IU/mL HBsAg was quantified using the ARCHITECT HBsAg assay (Abbott Laboratories, Chicago, IL, USA; LLoQ < 0.05 IU/mL). HBcrAg levels were assessed by chemiluminescent enzyme immunoassay using the LUMIPULSE G1200 analyser (Fujirebio Europe, Gent, Belgium; LLoQ 3.0 logU/mL) according to the manufacturer's instructions. cirB-RNA was quantified by HBV RNA investigational assay for use on the Cobas 6800/8800 molecular systems (Roche Diagnostics, Mannheim, Germany; LLoQ 10 cp/mL).

2.3 | Statistical Analysis

Categorical variables were described as absolute frequency and percentage, whereas continuous variables were reported

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as median and 25%–75% interquartile range (IQR). Statistical analysis was performed using SPSS version 29.0 and GraphPad Prisma version 10. For categorical variables, the Chi-square test or Fisher's exact test were used. For continuous data, comparisons between two independent groups were performed using *t*-test in case of normal distribution, Mann–Whitney *U*-test when data did not follow a normal distribution, and Kruskal–Wallis test, where more than two groups were compared. Kaplan–Meier curve and Log-Rank test were used to predict patients' outcomes. Statistical significance was set at p values <0.05.

3 | Results

3.1 | Baseline Characteristics of the Patients

We included 128 naïve patients followed for a median (IQR) of 5.1 (3.7–6.8) years. Detailed baseline patients' characteristics are depicted in Table 1. Analysis of demographic data revealed a significant age difference between the groups ($p=0.007$), with patients in group 1 being significantly older than those in group 3 (median age 44 [32–55] vs. 32 [23–46], $p=0.002$). Additionally, group 1 patients were more frequently Caucasian compared to those in groups 2 and 3 (82% vs. 63% vs. 54%, $p=0.017$).

3.2 | Detection of Biomarkers at Baseline

HBcrAg and cirB-RNA were more frequently detected among patients with higher qHBsAg levels (group 3: 27% and 19%, respectively). Conversely, HBcrAg and cirB-RNA were rarely positive among patients with qHBsAg <1000 IU/mL (group 1: 9% and 7%, respectively) (Table 1 and Figure 1A,B). Only 4 patients tested positive for both viral markers (cirB-RNA and HBcrAg): 2 patients from group 3 (5.4%), 1 from group 2 (5.7%) and 1 from group 1 (1.8%) (Figure 1C).

Patients testing positive for cirB-RNA had significantly higher HBV DNA levels compared to those who were cirB-RNA negative ($p<0.001$). However, similar HBV DNA levels were observed between patients with and without detectable HBcrAg. In contrast, HBcrAg positive patients showed higher levels of qHBsAg than patients with undetectable HBcrAg ($p=0.033$), while there was not a significant between cirB-RNA positive and negative patients.

3.3 | Correlation Between Clinical and Virological Outcomes and Viral Markers

When evaluating clinical and virological outcomes, 117 patients with a follow-up period of more than 1 year were included in the analysis: 52 (44.4%) from group 1, 32 (27.4%) from group 2, and 33 (28.2%) from group 3. The median follow-up was 5.1 (3.7–6.8) years. As shown in Figure 1D, most patients ($n=81$, 69.2%) remained in the same qHBsAg levels group throughout the study period. Impressively, 82.1% of patients demonstrated a reduction in qHBsAg levels >1 log IU/mL during the study period. Baseline qHBsAg <1000 IU/mL was the only factor significantly associated with qHBsAg levels reduction >1 log IU/mL (Table S1).

Only 10 patients (8.5%)—2 from group 2 and 8 from group 3—experienced an increase in HBV DNA above 2000 IU/mL, with just one progressing to HBeAg-negative chronic hepatitis (with ALT increase) requiring antiviral treatment initiation. None of the patients with qHBsAg below 1000 IU/mL experienced an increase in HBV DNA > 2000 IU/mL, whereas neither cirB-RNA nor HBcrAg status had an influence on this outcome (Figure S1).

During the study period, 11 patients (9.4%) achieved spontaneous functional cure (9 from group 1 and 2 from group 2). Baseline qHBsAg levels below 1000 IU/mL were highly predictive of HBsAg loss (Figure 1E, $p=0.004$). In contrast, none of the patients with a baseline qHBsAg >1000 IU/mL (group 3) achieved HBsAg loss. When assessing the impact of HBcrAg and cirB-RNA on patient outcomes, we found that their baseline levels had no influence on the likelihood of HBsAg loss (Figure 1F,G). Patients' characteristics according to virological outcomes are resumed in Table S2. In addition to HBsAg loss, we observed that 13 patients (11.1%) achieved qHBsAg <100 IU/mL (Figure S2), with baseline qHBsAg <1000 IU/mL being the only variable significantly associated with this outcome ($p=0.010$, Figure S3).

Finally, by the end of follow-up, 8 patients (4 from group 1, 3 from group 2, and 1 from group 3) experienced ALT elevation, with only one exceeding 2× ULN (84 IU/L). All were diagnosed with steatosis and/or alcohol consumption, whereas none had HBV-DNA elevation. Baseline cirB-RNA ($p=0.239$), HBcrAg ($p=0.979$), or qHBsAg ($p=0.567$) were not associated with ALT elevation.

4 | Discussion

The HBeAg-negative chronic infection phase typically follows a benign course and usually does not require antiviral treatment. However, accurate classification of patients into this phase of HBV infection requires prolonged observation and multiple outpatient clinic visits with laboratory assessments. Several studies indicate that, regardless of HBV genotype, the combination of qHBsAg levels <1000 IU/mL and HBV DNA <2000 IU/mL is as reliable as 1-year monitoring for diagnosing HBeAg-neg chronic infection [2, 12, 13]. Nonetheless, its role in predicting functional cure, except in the setting of NAs discontinuation, has not been well established yet.

In our study, we found that low HBsAg levels <1000 IU/mL, are independently associated with HBsAg loss at a median follow-up of 5 years. This is in line with other studies reporting higher chances of functional cure with low HBsAg levels [14, 15]. Additionally, these patients exhibited excellent outcomes; indeed, none experienced HBV DNA elevations above 2000 IU/mL or progressed to chronic hepatitis during follow-up. This confirms that a single measurement of qHBsAg can help identify HBeAg-neg chronic infection patients with minimal risk of disease progression, allowing for less frequent visits and, hence, reducing unnecessary healthcare costs. Conversely, patients with HBeAg-neg chronic infection and HBsAg levels above 1000 IU/mL would require more frequent monitoring, as their outcomes cannot be predicted based solely on HBsAg levels. However, it is important to note that while HBV DNA elevations

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TABLE 1 | Baseline patients' characteristics according to baseline qHBsAg levels, HBcrAg, and cirB-RNA detectability.

Naïve HBeAg-negative chronic infection patients $n = 128$								
Variables, n (%), median (IQR)	**Group 1 HBsAg 10–1000 IU/mL $n = 56$**	**Group 2 HBsAg 1000–10 000 IU/mL $n = 35$**	**Group 3 HBsAg >10 000 IU/mL $n = 37$**	**Variables, n (%), median (IQR)**	**HBcrAg pos $n = 18$**	**HBcrAg neg $n = 110$**	**cirB-RNA pos $n = 17$**	**cirB-RNA neg $n = 111$**
Female sex	22 (39.3)	20 (57.1)	15 (40.5)	Female sex	11 (15.5)	60 (84.5)	9 (12.7)	62 (87.3)
Age (years)[a]	44.5 (32–55)	41 (27–47)	32 (23–46.5)	Age (years)	32.5 (24–41.5)	40 (30–50)	43 (27.5–56.5)	38 (29–49)
Ethnicity[c]						*Ethnicity*[d]		
Caucasian	46 (82.1)	22 (62.9)	20 (54.1)	Caucasian	9 (50)	79 (71.8)	11 (64.7)	77 (69.4)
Asian	7 (12.5)	5 (14.3)	8 (21.6)	Asian	6 (33.3)	14 (12.7)	1 (5.9)	19 (17.1)
African	2 (3.6)	6 (17.1)	3 (8.1)	African	3 (16.7)	8 (7.3)	4 (23.5)	7 (6.3)
Others	1 (1.8)	2 (5.7)	6 (16.2)	Others	0	9 (8.2)	1 (5.9)	8 (7.2)
Follow-up (years)	13.9 (4.5–24.6)	6.7 (3.7–11.8)	7.9 (3.1–18.4)	Follow-up (years)	3.7 (1.1–7.3)	4.9 (3.6–6.5)	5.4 (4.4–6.6)	4.7 (2.9–6.7)
ALT (IU/L)	21 (16–30)	19 (16–25)	23 (19–28)	ALT (IU/L)	20 (15–29)	21 (16–28)	21 (15–28)	21 (16–28)
Serological viral markers at baseline						Serological viral markers at baseline		
qHBsAg (IU/mL)	110 (37–522)	3749 (2352-5809)	22 413 (14934-33 558)	qHBsAg (IU/mL)[d]	15 081 (872–22 514)	1464 (195–9394)	7106 (815–18 006)	1569 (182–11 544)
HBV DNA (IU/mL)[b]	225 (37–651)	494 (114–1215)	475 (166–823)	HBV DNA (IU/mL)[e]	215 (51–590)	423 (89–834)	1290 (765–1710)	268 (59–619)
cirB-RNA, pos	4 (7.1)	6 (17.1)	7 (18.9)					
HBcrAg, pos[c]	5 (8.9)	3 (8.6)	10 (27)					

[a]Difference between groups 1 and 3 is statistically significant, $p < 0.05$.
[b]Difference between group 1 and 2 and group 1 and 3 is statistically significant, $p < 0.05$.
[c]Difference between group 1, 2, and 3 is statistically significant, $p < 0.05$.
[d]Difference between HBcrAg pos/neg is statistically significant, $p < 0.05$.
[e]Difference between cirB-RNA pos/neg is statistically significant, $p < 0.05$.

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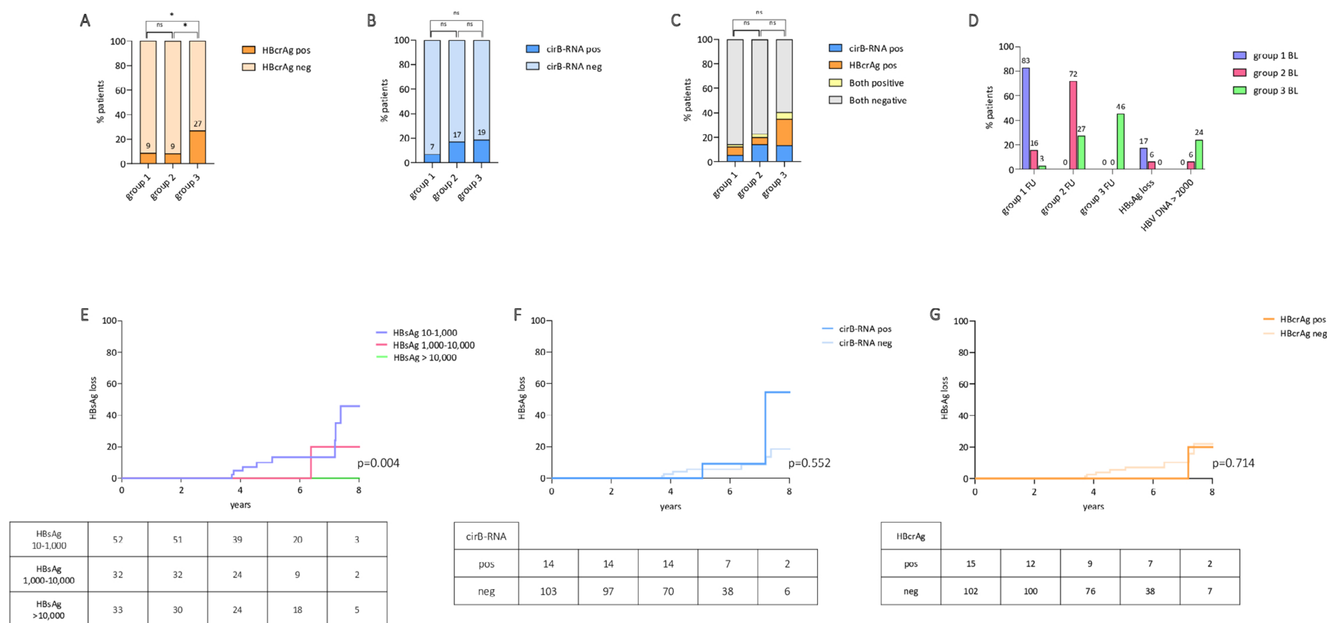

HBsAg 10-1,000	52	51	39	20	3
HBsAg 1,000-10,000	32	32	24	9	2
HBsAg >10,000	33	30	24	18	5

cirB-RNA					
pos	14	14	14	7	2
neg	103	97	70	38	6

HBcrAg					
pos	15	12	9	7	2
neg	102	100	76	38	7

FIGURE 1 | Distribution of viral markers according to patient groups (Group 1: QHBsAg 10–1000 IU/mL, Group 2: QHBsAg 1000–10 000, Group 3: QHBsAg > 10 000) and clinical outcomes. (A) Baseline distribution of detectable and undetectable HBcrAg. (B) Baseline distribution of detectable and undetectable cirB-RNA. (C) Baseline distribution of both detectable and undetectable cirB-RNA and HBcrAg. (D) Patients' transition between groups, to HBsAg loss and to HBV DNA increase > 2000 IU/mL according to baseline qHBsAg. Kaplan–Meier curve for HBsAg loss during follow-up according to baseline HBsAg levels (E), cirB-RNA (F) and HBcrAg (G). *$p < 0.05$.

were observed in 22% of individuals with qHBsAg levels exceeding 10 000 IU/mL, the transition to HBeAg-neg chronic hepatitis was a rare event (only one patient had concomitant ALT elevation and required initiation of antiviral treatment). Moreover, over 80% of our patients achieved a reduction in qHBsAg levels exceeding 1 log IU/mL during the study period, with 11.1% reaching qHBsAg values below 100 IU/mL. A baseline qHBsAg threshold of 1000 IU/mL was the sole predictor of reduction in qHBsAg below 100 IU/mL, whereas neither cirB-RNA nor HBcrAg proved useful in predicting these outcomes.

Novel viral biomarkers, namely cirB-RNA and HBcrAg, have demonstrated promising results in discerning between active and inactive disease in both HBeAg-positive and -negative patients [16, 17]. Yet, their role in delineating clinical and virological outcomes, particularly within the specific cohort of untreated HBeAg-neg chronic infection patients, remains to be clarified. A large multicentre study revealed that higher levels of HBcrAg and HBV RNA were associated with elevated ALT, as well as higher APRI and FIB-4 [10]. However, they failed to enhance the accuracy of conventional viral markers in distinguishing between different phases of chronic hepatitis B. Loggi et al. [18] observed lower HBcrAg detection rates in HBeAg-neg chronic infection patients compared to those with chronic hepatitis. However, they also revealed that within the HBeAg-neg chronic infection patients' subgroup, those exhibiting fluctuating viral loads, occasionally exceeding 2000 IU/mL, had higher HBcrAg detection rates. Despite this difference, both subgroups exhibited similar clinical outcomes.

Importantly, in our cohort, neither cirB-RNA nor HBcrAg appeared to differentiate HBeAg-neg chronic infection patients with a different natural history.

The limited ability of cirB-RNA and HBcrAg to predict HBsAg loss in HBeAg-neg chronic infection patients may be attributed to their low prevalence in this specific patients' cohort due to the nature of these markers. They primarily reflect the cccDNA transcriptional activity [3], which is generally down-regulated in HBeAg-neg chronic infection patients, indicative of a more controlled or inactive state of the infection [19]. Indeed, in these individuals, HBsAg primarily originates from integrated viral DNA [20]. Additionally, both markers were rarely positive simultaneously in the same patient, so the significance of a single positive marker still needs further clarification.

Our study's strength relies on the use of a cohort of well-characterised naïve HBeAg-neg chronic infection patients, with an extensive clinical follow-up period. We employed automated assays for cirB-RNA quantification and stipulated a minimum follow-up period of 12 months to meticulously document patient evolution according to liver tests, viral load, and qHBsAg analysis. However, our study also has limitations. Due to its retrospective nature, it was possible to assess follow-up cirB-RNA and HBcrAg only in a subset of patients. In addition, a longer follow-up would have been optimal to strengthen our conclusions.

In summary, we have confirmed that patients with HBeAg-neg chronic infection generally have a favourable prognosis regardless of HBsAg levels or the presence of HBcrAg or cirB-RNA. Our data show that single-point cirB-RNA and HBcrAg do not predict clinical and virological outcomes in HBeAg-negative chronic infection untreated patients. Conversely, a single HBsAg value below 1000 IU/mL can reliably predict HBsAg loss and identify patients at minimal risk of disease progression, allowing for extended follow-up intervals and, thus, significant cost savings.

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Author Contributions

Contributed to the study concept and design: Sergio Rodrìguez-Tajes, Sabela Lens, Sofía Pérez-del-Pulgar, Xavier Forns. Patient selection/inclusion: Anna Pocurull, Sergio Rodrìguez-Tajes, Zoe Mariño, Sabela Lens, Xavier Forns. Acquired data: Thais Leonel, Sergio Rodrìguez-Tajes, Anna Pocurull, Maria Saez-Palma, Sofía Pérez-del-Pulgar, Ariadna Rando-Segura, David Tabernero, Maria Francesca Cortese, Sabela Lens, Xavier Forns. Contributed to analysis and interpretation of data: Sara Battistella, Thais Leonel, Sergio Rodrìguez-Tajes, Anna Pocurull, Ariadna Rando-Segura, Sofía Pérez-del-Pulgar, Sabela Lens, Xavier Forns. Drafted the manuscript: Sara Battistella, Sabela Lens, Thais Leonel, Sofía Pérez-del-Pulgar, Xavier Forns. Contributed to critical revisions and approved the final manuscript: all authors.

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Ethics Statement

This study was authorised by the Ethical Committee of Hospital Clinic.

Consent

All participants gave their informed consent.

Conflicts of Interest

Z.M.: Speaker fees for Orphalan and Gilead; consultancy fees for Orphalan, Alexion, DeepGenomics; grants from Gilead. X.F. has acted as an advisor for Gilead. S.L. has received lecture and consulting fees from Gilead, Abbvie, Roche, and GSK, and research grants from Gilead. S.R.-T. has received lecture fees from Gilead and Abbvie. All other authors report no potential conflicts of interest. Please refer to the accompanying ICMJE disclosure forms for further details.

Data Availability Statement

Data are available from the corresponding authors (S.L., slens@clinic.cat; S.P.P., sofiapp@recerca.clinic.cat) upon reasonable request.

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