


ORIGINAL ARTICLE

HBsAg isoform dynamics during NAP-based therapy of HBeAg-negative chronic HBV and HBV/HDV infection

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Abstract

Nucleic acid polymers block the assembly of hepatitis B virus (HBV) subviral particles, effectively preventing hepatitis B surface antigen (HBsAg) replenishment in the circulation. Nucleic acid polymer (NAP)-based combination therapy of HBV infection or HBV/hepatitis D virus (HDV) co-infection is accompanied by HBsAg clearance and seroconversion, HDV-RNA clearance in co-infection, and persistent functional cure of HBV (HBsAg < 0.05 IU/ml, HBV-DNA target not detected, normal alanine aminotransferase) and persistent clearance of HDV RNA. An analysis of HBsAg isoform changes during quantitative HBsAg declines (qHBsAg), and subsequent treatment-free follow-up in the REP 301/REP 301-LTF (HBV/HDV) and REP 401 (HBV) studies was conducted. HBsAg isoforms were analyzed from frozen serum samples using Abbott Research Use Only assays for HBsAg isoforms (large [L], medium [M], and total [T]). The relative change over time in small HBsAg relative to the other isoforms was inferred by the change in the ratio over time of T-HBsAg to M-HBsAg. HBsAg isoform declines followed qHBsAg declines in all participants. No HBsAg isoforms were detectable in any participants with functional cure. HBsAg declines > 2 log₁₀ IU/ml from baseline were correlated with selective clearance of S-HBsAg in 39 of 42 participants. Selective S-HBsAg decline was absent in 9 of 10 participants with HBsAg decline < 2 log₁₀ IU/ml from baseline. Mild qHBsAg rebound during follow-up <10 IU/ml consisted mostly of S-HBsAg and M-HBsAg and not accompanied by significant covalently closed circular DNA activity. **Conclusion:** The faster observed declines in S-HBsAg indicate the selective clearance of subviral particles from the circulation, consistent with previous mechanistic studies on NAPs.

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Trace HBsAg rebound in the absence of HBV DNA may reflect HBsAg derived from integrated HBV DNA and not rebound of viral infection.

INTRODUCTION

Chronic hepatitis B virus (HBV) infection is accompanied by fibrosis, cirrhosis, and hepatocellular carcinoma.^[1] Affecting approximately 300 million people worldwide,^[2] chronic HBV infection is responsible for 887,000 deaths annually.^[3] Hepatitis D virus (HDV) is a satellite infection of HBV, which requires hepatitis B surface antigen (HBsAg) to form its envelope.^[4] Co-infection with HDV affects 20–40 million people^[5,6] worldwide and accelerates the progression of liver disease.^[7] In both conditions, HBsAg is the most abundant viral antigen, >99.99% of which is derived from noninfectious subviral particles (SVPs).^[8] SVPs are produced independently from viral replication and independently from covalently closed circular DNA (cccDNA) via integrated HBV DNA.^[8] The SVP assembly/secretory pathway is also involved in HDV envelopment and secretion.^[8]

HBsAg is linked to inhibition of the innate and adaptive immune responses to HBV infection.^[8] These immunoinhibitory activities of HBV are a major factor contributing to the maintenance of chronic HBV-mediated hepatitis. Consistent with these effects, clearance of HBsAg during therapy is the only currently established endpoint that reliably predicts functional cure of HBV,^[9,10] in which viremia remains controlled (HBsAg < 0.05 IU/ml, undetectable HBV DNA) and liver function remains normal (alanine aminotransferase [ALT] < upper limit of normal) in the absence of any therapy.^[9,11–14] As such, the inability of existing therapies to achieve HBsAg loss during therapy in more than a small fraction of patients^[15,16] is an important limitation in achieving functional cure with HBV and HDV infection.

In the open reading frame of the HBsAg messenger RNA, three in-frame start codons lead to the production of three HBsAg isoforms: S-HBsAg, M-HBsAg, and L-HBsAg (where S indicates selective, M indicates medium, and L indicates large).^[17] S-HBsAg contains four membrane-spanning domains as well as the “a”-determinant, the primary antigenic site of HBsAg.^[18] In addition to these domains, M-HBsAg contains an additional amino terminal preS2 sequence, and L-HBsAg contains both amino terminal preS1 and preS2 sequences.^[17] Both preS1 and preS2 sequences are required for virion assembly^[17,19] but are dispensable for spherical SVP^[20,21] and HDV^[22,23] assembly. Additionally, the preS1 sequence uniquely found in L-HBsAg contains the interaction domain required

for sodium taurocholate cotransporting peptide-dependent viral entry.^[24–26] In spherical SVPs, S-HBsAg accounts for about 95% of HBsAg, with only minor traces of L-HBsAg (<1%) present.^[27] However, in virions and SVP filaments, L-HBsAg is substantially enriched (~25%).^[17,27] This enrichment is likely driven by L-HBsAg by interaction with γ 2-adaptin in post-endoplasmic reticulum vesicles that transit to the multivesicular body during viral morphogenesis.^[28] With spherical SVPs forming the bulk of circulating HBsAg and consisting mostly of S-HBsAg, this isoform is the major circulating HBsAg isoform. However, while M-HBsAg and L-HBsAg are found during acute and chronic infection, their levels have been proposed to drop significantly in inactive HBV carriers,^[29] consistent with the reduced viremia observed in this patient population. Additionally, a recent study has observed that selective declines in M-HBsAg and L-HBsAg precede HBsAg loss during nucleos(t)ide analog (NUC) therapy in HBeAg-positive HBV infection.^[30]

Nucleic acid polymers (NAPs) selectively target the assembly and secretion of spherical SVPs^[31,32] from both cccDNA and integrated HBV DNA,^[33] effectively blocking the replenishment of HBsAg in the circulation. This effect is accompanied by declines in intracellular HBsAg^[31,32] and clearance of HBsAg from the liver.^[34,35] In clinical studies, NAP monotherapy is accompanied by rapid declines of HBsAg to levels < 0.05 IU/ml, HBsAg seroconversion, and rapid declines in HBV RNA and HBV DNA.^[36,37] In HBeAg-positive chronic infection, rapid clearance and seroconversion of HBeAg are also observed,^[36] and in HBV/HDV co-infection, additional rapid clearance of HDV RNA occurs.^[33] When combined with pegylated interferon (pegIFN) or tenofovir disoproxil fumarate (TDF) and pegIFN, NAP-based combination therapy leads to high rates of HBsAg loss (<0.005 IU/ml) and seroconversion, host-mediated transaminase flares, high rates of cccDNA silencing, and high rates of functional cure of HBV with persistent HBsAg seroconversion^[38–40] and persistent undetectable HDV RNA (in co-infected individuals).^[41] A retrospective analysis of changes in HBsAg isoform composition during therapy and follow-up from NAP-based combination therapy was performed to establish the pattern of response in each HBsAg isoform during therapy and treatment-free follow-up. This analysis included all participants with HBeAg-negative HBV infection in the REP 401 study^[38] and HBeAg-negative HBV/HDV co-infection in the REP 301^[33]/REP 301-LTF studies.^[41]

METHODS

The study design for the REP 301, REP 301-LTF, and REP 401 studies are presented in [Figure 1](#). The REP 301 study was a nonrandomized, noncontrolled study in 12 patients with HBeAg-negative chronic HBV/HDV co-infection, in which 15 weeks of REP 2139-Ca was followed by 15 weeks of REP 2139-Ca and pegIFN followed by 33 weeks of pegIFN. An initial 24-week treatment-free follow-up was followed by an additional 3-year follow-up in the REP 301-LTF study with visits every 6 months. In the REP 401 study, 40 participants with chronic HBeAg-negative HBV infection received 24 weeks of TDF followed by randomization to receive either TDF + pegIFN or TDF + pegIFN + NAPs (REP 2139-Mg or REP 2165-Mg) for 48 weeks. All participants receiving 24 weeks of TDF + pegIFN were switched to TDF + pegIFN + NAPs for 48 weeks for futility. Treatment-free follow-up was 48 weeks. All procedures were conducted in accordance with the National Health Authority and Ethics Committee of the Republic of Moldova.

Assay linearity tests with patient samples

Frozen serum samples ($n = 1153$) from all 52 participants in the REP 301/REP 301-LTF and REP 401 studies were analyzed using the Abbott research use only (RUO) assays for HBsAg isoforms (large [L], medium [M] and total [T]) as previously described.^[42] Each of the three RUO HBsAg isoforms assays is an automated chemiluminescent microparticle immunoassay that uses mouse monoclonal antibodies to specifically capture HBsAg from the serum. Following a wash step, L-HBsAg, M-HBsAg, and T-HBsAg isoforms are revealed using acridinium labelled mouse monoclonal

antibodies specific for PreS1, PreS2, and S-HBsAg, respectively, and measured in relative light units. Signal to noise was determined for each isoform assay by running a panel of 50 HBsAg-negative serum samples, and a cutoff (S/Co) was set at 2 S/N. The relative change over time in S-HBsAg relative to the other isoforms was inferred by the change in the ratio over time of T-HBsAg to M-HBsAg S/Co results. Time points were when any of the measurements of T-HBsAg and M-HBsAg with S/Co < 1 were excluded from the ratio/trend analysis of S-HBsAg. HBsAg isoform datapoints from REP 401 participants 02-057 (study week 75) and 02-023 (study week 87) were removed from the analysis data set, as they were inconsistent with quantitative HBsAg (qHBsAg) values from these timepoints and from isoform datapoints both before and after in these participants. Statistical analysis was performed by t-test, single-factor analysis of variance (ANOVA), regression ANOVA, or χ^2 test where appropriate. Statistical significance was considered met with $p < 0.05$.

RESULTS

HBsAg isoform results (T-HBsAg, M-HBsAg, and L-HBsAg) in the current study were compared with previously published qHBsAg and alanine aminotransferase data.^[33,38,41] In the REP 301 study (HBV/HDV co-infection), individual participant responses of T-HBsAg, M-HBsAg, and L-HBsAg declined similarly to those of qHBsAg early during REP 2139-Ca monotherapy ([Figure 2](#)). However, at the end of REP 2139 monotherapy, small amounts of qHBsAg, T-HBsAg, and M-HBsAg persisted in 4 participants, whereas L-HBsAg was not detectable (S/Co < 1) ([Figure 2A](#), boxes). With the introduction of interferon and the onset of strong transaminase flares in these 4 participants, the residual

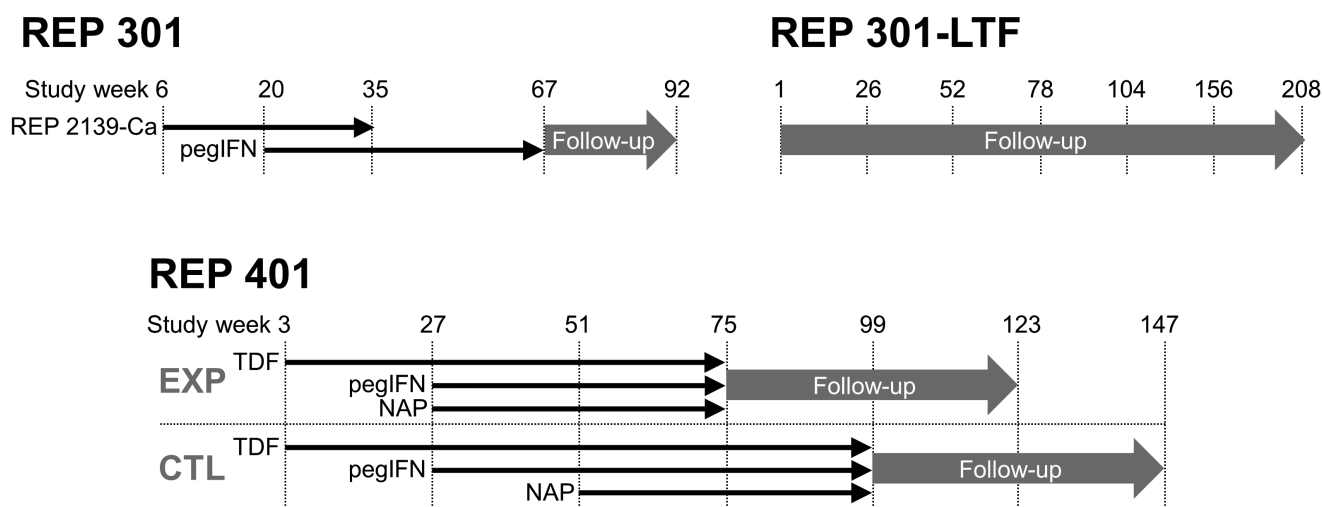


FIGURE 1 Designs of the REP 301 and REP 301-LTF studies (top) and the REP 401 study (bottom). CTL, control; EXP, experimental; NAP, nucleic acid polymer; pegIFN, pegylated interferon; TDF, tenofovir disoproxil fumarate

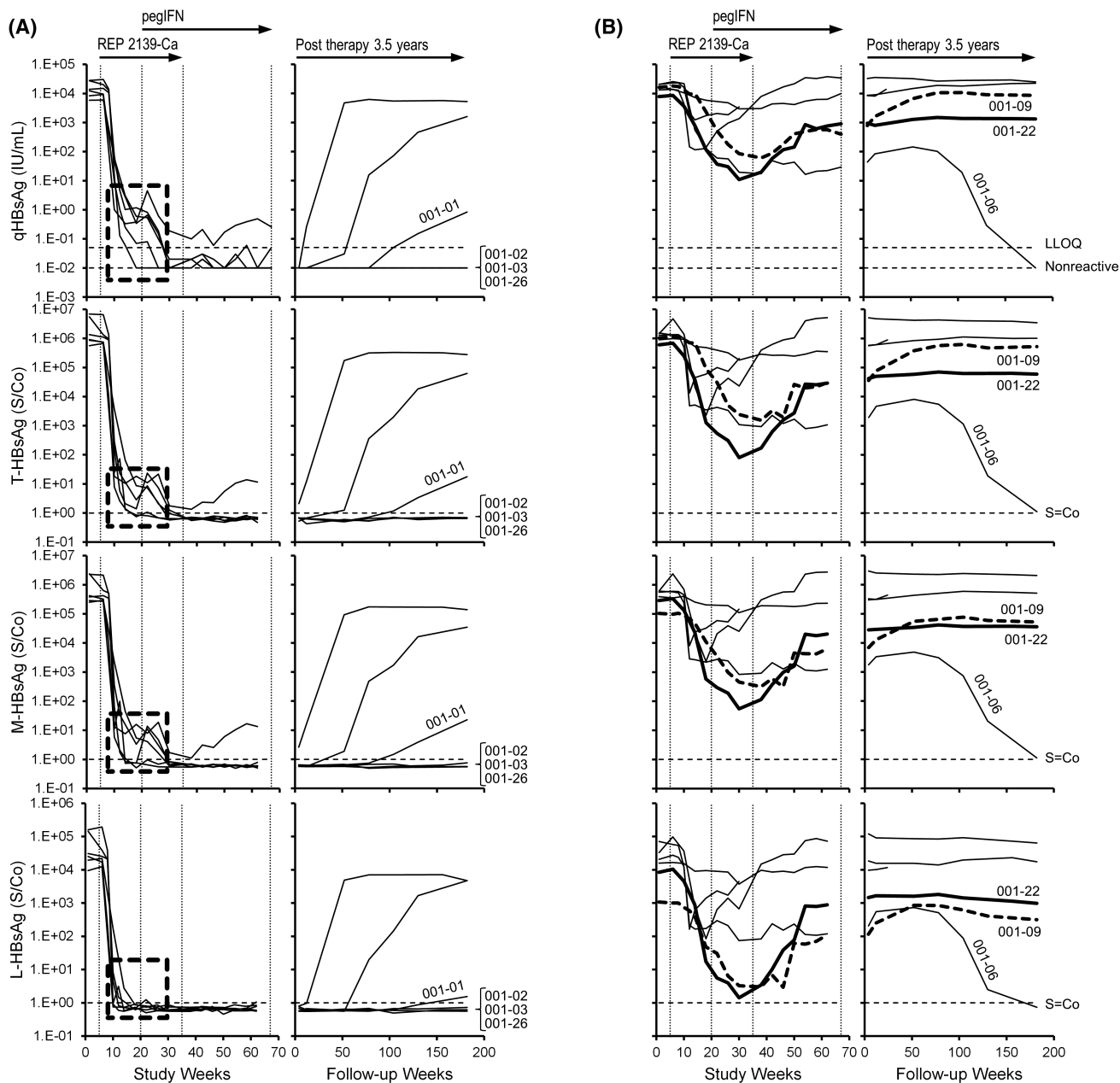


FIGURE 2 Individual participant dynamics of total hepatitis B surface antigen (T-HBsAg), medium HBsAg (M-HBsAg), and large HBsAg (L-HBsAg) isoforms during therapy and follow-up in the REP 301/301-LTF studies. (A,B) Participants are grouped according to strong HBsAg decline (A) or more moderate HBsAg decline (B). Dashed boxes in (A) highlight the isoform composition at the end of REP 3139-Ca monotherapy. The 2 participants with moderate quantitative HBsAg (qHBsAg) response, in whom clearance of L-HBsAg appeared more efficient (001-09 and 001-22), are highlighted in bold in (B). The qHBsAg data were previously published.^[33,41] LLOQ, lower limit of quantitation

qHBsAg, T-HBsAg, and M-HBsAg declined or became undetectable in all of these participants (Figure 3). All isoform trends followed qHBsAg throughout the rest of therapy and follow-up (Figure 2) except for 2 participants with moderate qHBsAg response (001-09 and 001-22), where clearance of L-HBsAg appeared more efficient (Figure 2B). In the 4 participants with qHBsAg < 0.05 IU/ml at the end of follow-up, T-HBsAg, M-HBsAg, and L-HBsAg were also all undetectable. In 1 participant (001-01), rebound in qHBsAg was mirrored

by rebound in T-HBsAg and M-HBsAg but rebound of L-HBsAg was much weaker (Figure 2A).

In the REP 401 study (HBV infection), strong and rapid qHBsAg declines were observed only following the introduction of NAPs (Figure 4A) and became <1 IU/ml in 29 of 40 participants during 48 weeks of therapy with TDF + pegIFN + NAPs (Figure 4B). In 1 control participant receiving only TDF + pegIFN (02-001), a more efficient clearance of L-HBsAg was observed (Figure 4A). In participants experiencing

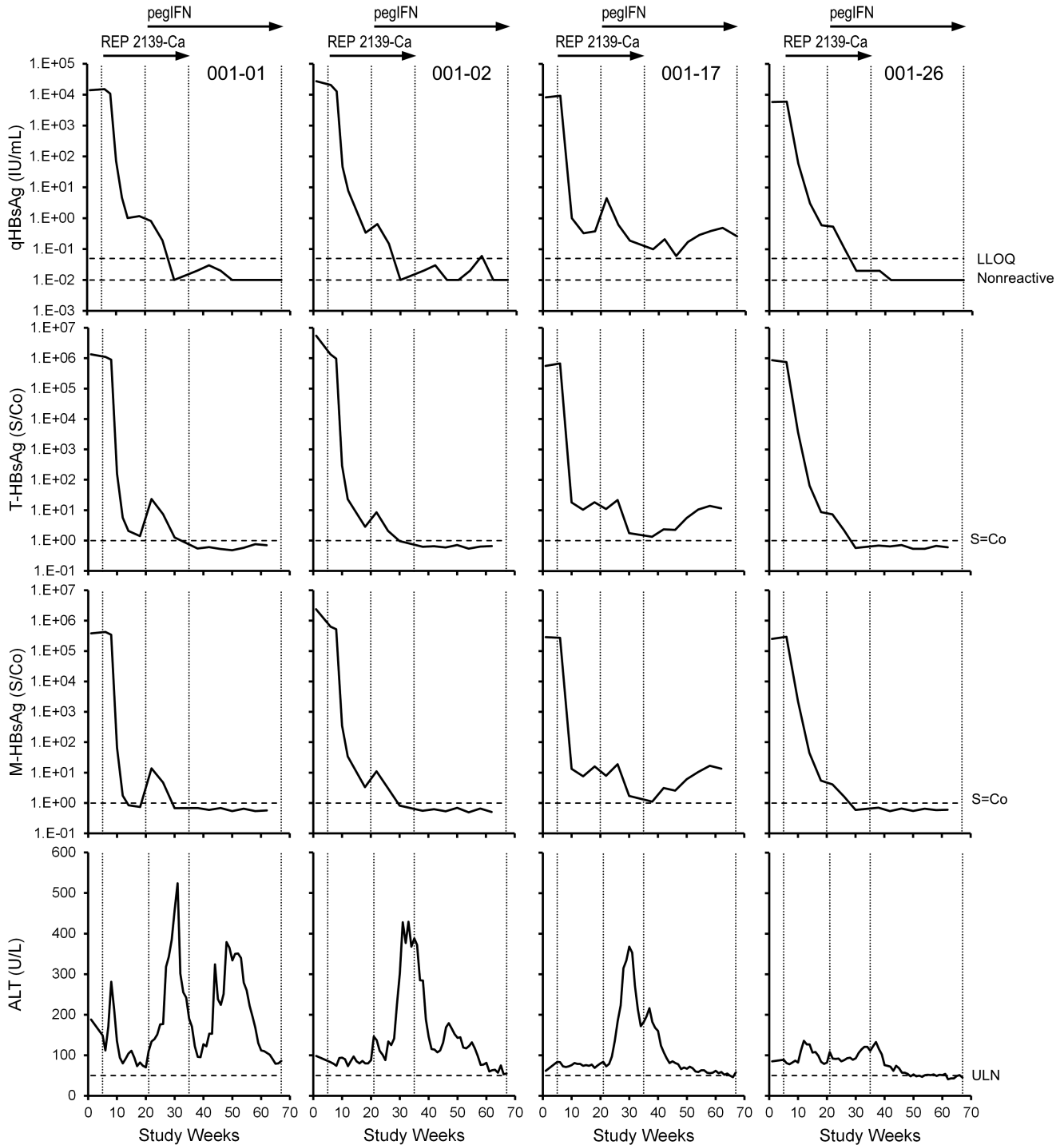


FIGURE 3 Individual participant dynamics of T-HBsAg and M-HBsAg isoforms and alanine aminotransferase (ALT) in the 4 REP 301 participants with residual qHBsAg present at the end of REP 2139-Ca monotherapy. The qHBsAg and alanine aminotransferase (ALT) data were previously published^[33]

qHBsAg decline to <1 IU/ml, declines in qHBsAg early during NAP exposure were mirrored by declines in T-HBsAg, M-HBsAg, and L-HBsAg; however, clearance of L-HBsAg tended to occur earlier (Figure 4B, boxes). Additionally, more efficient clearance of L-HBsAg in 1 participant with more moderate HBsAg response (01-075) was observed (Figure 4B).

During follow-up, 19 participants maintained qHBsAg < 1 IU/ml throughout follow-up (Figure 5A) but trace qHBsAg rebound observed in 3 of these participants appeared to be comprised primarily of T-HBsAg and M-HBsAg (Figure 5A). Delayed minor rebound in qHBsAg (~ 10 IU/ml) occurred in 4 participants and appeared to be comprised primarily of

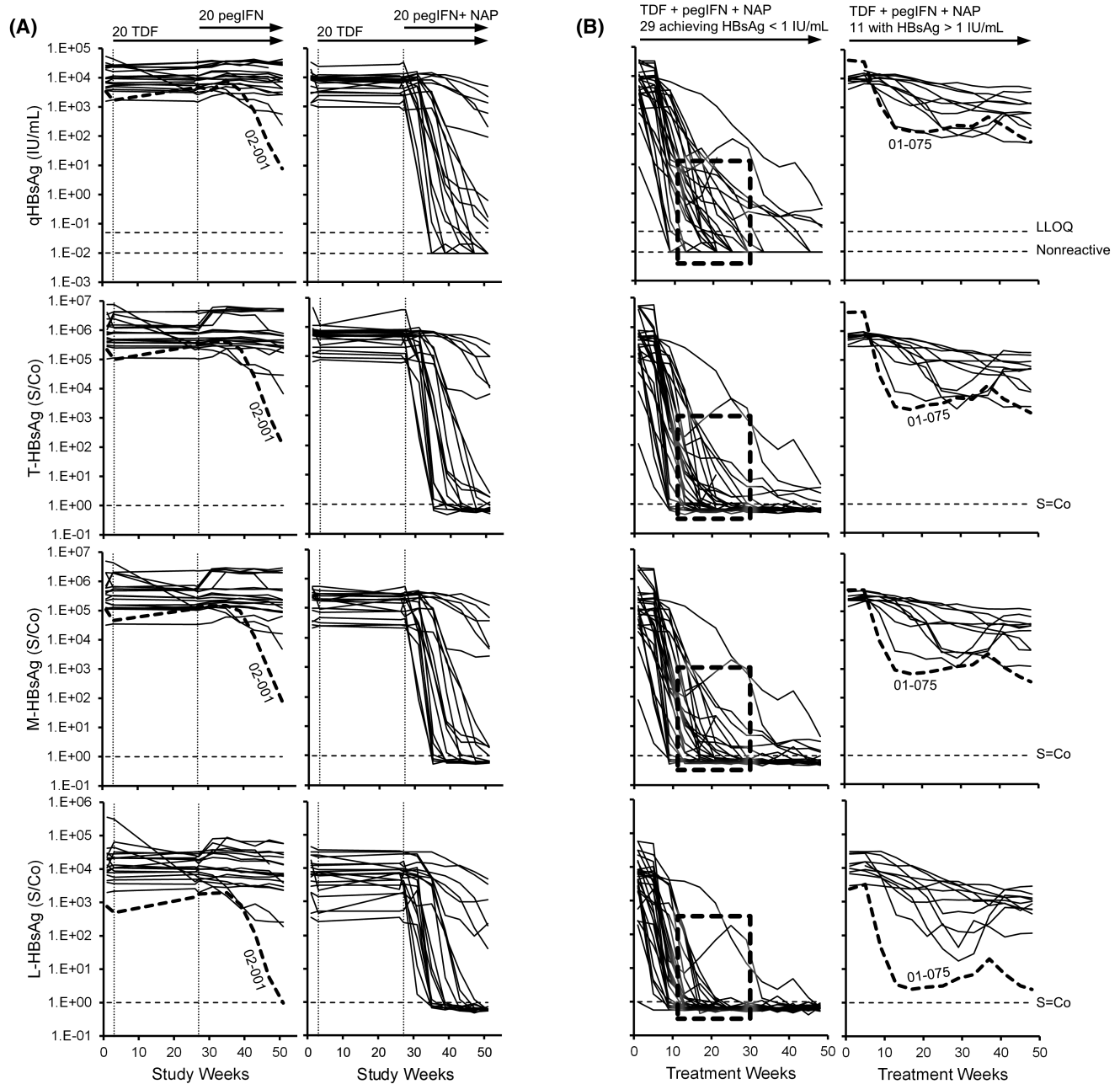


FIGURE 4 Individual participant dynamics of T-HBsAg, M-HBsAg, and L-HBsAg isoforms during therapy in the REP 401 study. (A,B) Dynamics during the initial 48 weeks of therapy in control and experimental groups (A) and during 48 weeks of TDF + pegIFN + NAP therapy (B). Dashed boxes in (B) highlight the more efficient clearance of L-HBsAg during therapy in participants achieving qHBsAg < 1 IU/ml during therapy. Isoform composition at the end of REP 3139-Ca monotherapy. The 2 participants in whom clearance of L-HBsAg appeared more efficient (01-075 and 02-001) are highlighted in bold. The qHBsAg data were previously published^[38]. TDF, tenofovir disoproxil fumarate

T-HBsAg and M-HBsAg (Figure 5B). Early qHBsAg rebound > 1 IU/ml was observed in the remaining 17 participants in whom qHBsAg rebound was mirrored by T-HBsAg, M-HBsAg, and L-HBsAg (Figure 5C). In the 8 participants in the REP 301-LTF and REP 401 studies, in whom trace qHBsAg rebound appeared devoid of L-HBsAg, the transcriptional activity/prevalence of cccDNA appeared to be either very low or absent (Table 1).

As a prerequisite for examining selective declines in S-HBsAg, linearity assessment was performed for the T-HBsAg, M-HBsAg, and L-HBsAg assays on two external serum samples from Red Cross patients with HBV infection and one baseline serum sample from a REP 301 (01-002) and a REP 401 (01-024) participant (Figure S1). These analyses demonstrated good linearity in all three assays and stable T-HBsAg-to-M-HBsAg ratios with qHBsAg dilutions down to

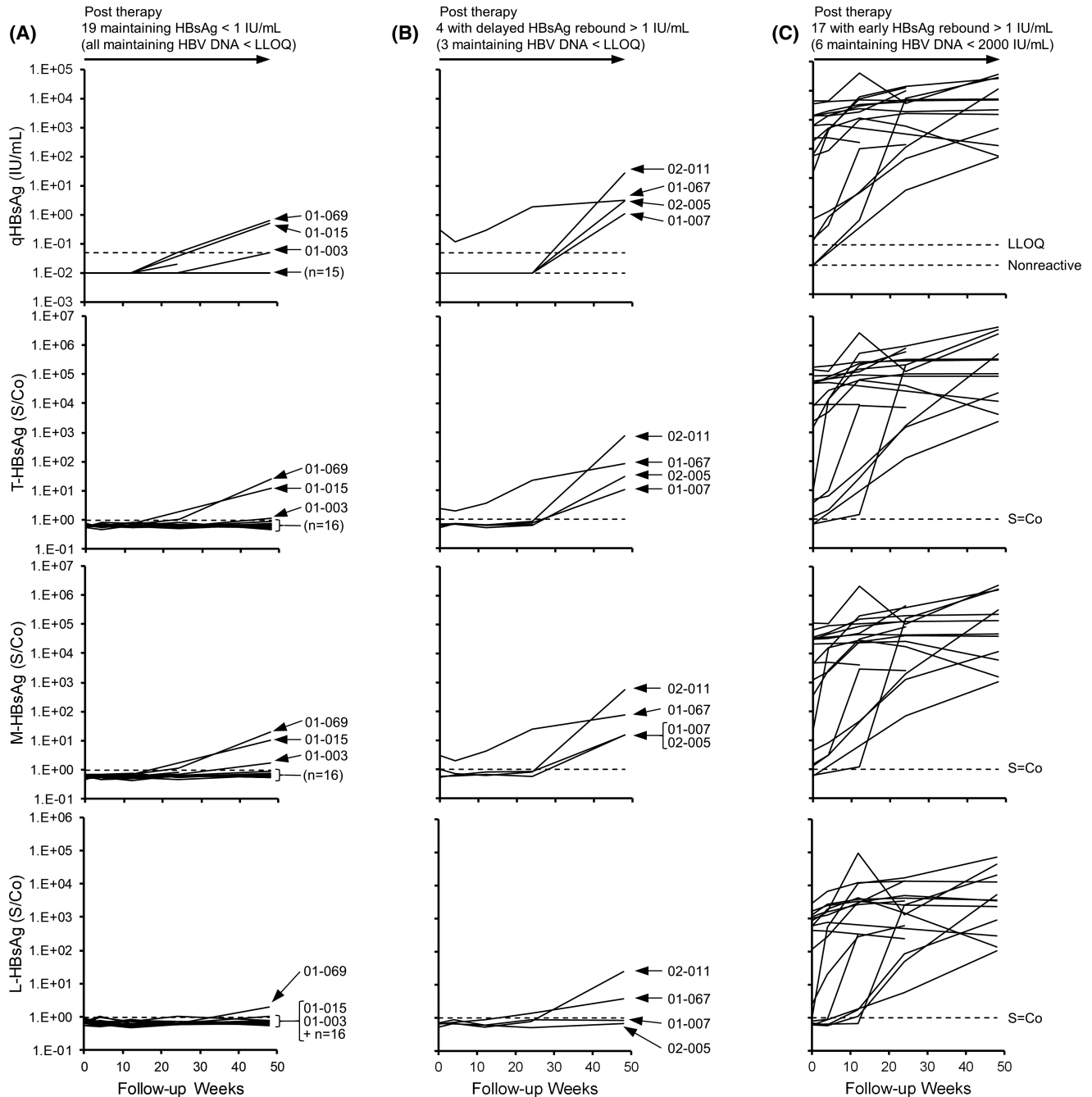


FIGURE 5 Individual participant dynamics of T-HBsAg, M-HBsAg, and L-HBsAg isoforms in the REP 401 study during follow-up. (A–C) Participants maintaining qHBsAg < 1 IU/ml (A), with delayed rebound of HBsAg > 1 IU/ml (B) or early rebound of HBsAg > 1 IU/ml (C). Individual participants in whom HBsAg rebound appeared to consist primarily of T-HBsAg and M-HBsAg are identified. The qHBsAg data were previously published^[38]

about 5 IU/ml, indicating that selective declines in S-HBsAg could be examined by comparing the change over time of T-HBsAg to M-HBsAg (T:M). Analyses of changes in T:M over time in all 52 participants in the REP 301/301-LTF and REP 401 studies revealed two distinct patterns. In participants with minimal or very slow qHBsAg response (Figure 6A,C,E), very little change in T:M was observed, indicating that no significant selective reduction in S-HBsAg was

occurring. In participants with strong qHBsAg response (Figure 6B,D,F), rapid declines in the T:M ratio were observed co-incident with rapid reduction in qHBsAg. This indicated that the strong declines in qHBsAg in these participants was correlated with a more rapid decline in S-HBsAg than M-HBsAg or L-HBsAg. An analysis of selective S-HBsAg decline versus qHBsAg response in all 52 participants (Table 2) indicated that S-HBsAg response was significantly

TABLE 1 Markers of Covalently Closed Circular DNA activity at end of follow-up in participants with qHBsAg rebound <10 IU/ml

Participant	HBV DNA ^a	HBV pgRNA ^a	HBcrAg ^a
001-01	TND	72 U/ml	<LLOQ
01-069	<LLOQ	<LLOQ	<LLOQ
01-015	TND	TND	<LLOQ
01-003	TND	<LLOQ	<LLOQ
02-011	<LLOQ	<LLOQ	<LLOQ
01-067	41 IU/mL	<LLOQ	<LLOQ
02-005	<LLOQ	<LLOQ	<LLOQ
01-007	TND	TND	<LLOQ

^aPreviously published.^[38]

TND, target not detected.

correlated ($p < 0.01$) with HBsAg response $> 2 \log_{10}$ IU/ml from baseline.

The recent demonstration of the potential for using HBsAg isoform composition as a means to predict HBsAg loss during NUC therapy in HBeAg-positive chronic HBV infection^[30] led to the analysis of the relationship between baseline qHBsAg, T-HBsAg, M-HBsAg, and L-HBsAg, and qHBsAg reduction during NAP-based combination therapy (greater vs. $< 2 \log_{10}$ decline from baseline) and HBV therapeutic outcome after NAP-based combination therapy (rebound, virologic control, or functional cure) in both the REP 301/301-LTF and REP 401 studies (Figure S2). There were no statistically significant differences between baseline qHBsAg, T-HBsAg, M-HBsAg, or L-HBsAg and HBsAg response during therapy or HBV therapeutic outcome during follow-up.

DISCUSSION

Declines in qHBsAg observed during NAP-based combination therapy in the REP 301/301-LTF and REP 401 studies included declines in all HBsAg isoforms and were correlated with the introduction of NAPs. A more rapid clearance of S-HBsAg was observed in 39 of 42 participants with strong qHBsAg declines ($> 2 \log_{10}$ IU/ml from baseline) and is consistent with the selective effect of NAPs on spherical SVP assembly and secretion previously published.^[31,32] Selective S-HBsAg decline was absent in 9 of 10 participants with moderate ($< 2 \log_{10}$ IU/ml from baseline) qHBsAg decline, consistent with the moderate qHBsAg response typically observed with TDF + pegIFN,^[15] further suggesting not only that the antiviral effect of NAPs was attenuated in these patients but also that inhibiting SVP assembly and release is required for strong and rapid HBsAg decline. Although assay linearity studies showed good linear reduction in signal across the range of HBsAg concentrations tested, the lack of suitable reference

standards for the M and L proteins makes the sensitivities of these assays impossible to determine at the current time. Additionally, the T, M, and L isoform assays are not quantitative, whereas the qHBsAg assay is a quantitative assay standardized to the World Health Organization International HBsAg reference. As such, the possibility L-HBsAg may be present in those patients with no detectable L-HBsAg during the follow-up cannot be excluded.

The basis for the reduced HBsAg response to NAPs in a small subset of participants^[33,36,38] is not fully understood but does not appear to be due to reduced uptake into hepatocytes, as HDV-RNA responses are similar in participants co-infected with HBV/HDV, regardless of HBsAg response.^[33] In earlier studies, mild HBsAg response was rescued by increased frequency dosing of NAPs,^[36] suggesting that trafficking of NAPs to the ERGIC (the site of SVP morphogenesis^[19]) may be attenuated in these participants and improved by more NAP frequent uptake into hepatocytes.

No HBsAg isoforms were detected at the end of follow-up in all participants who achieved functional cure of HBV. However, the trace qHBsAg rebound (< 10 IU/ml) in 8 participants during follow-up appeared to consist primarily of S-HBsAg and M-HBsAg. Moreover, markers for cccDNA activity were either very low or absent in these participants at the end of follow-up. Although the production of S-HBsAg and M-HBsAg is universally preserved in integrated HBV DNA, the production of L-HBsAg is destroyed by disruption of the preS1 region in a significant minority of HBV-DNA integrations.^[23,43,44] Thus, the selective rebound of S-HBsAg and M-HBsAg in these participants may be derived from integrated HBV DNA not capable of producing L-HBsAg.

No correlation was found between baseline levels of HBsAg isoforms and HBsAg decline during NAP-based therapy or therapeutic outcomes during follow-up. However, in addition to the selective S-HBsAg declines observed early after the introduction of NAPs in the REP 301/301-LTF and REP 401 studies, overall HBsAg clearance as qHBsAg became < 10 IU/ml appeared to be more efficient for L-HBsAg than the other HBsAg isoforms. Although this selective effect on L-HBsAg clearance appears reminiscent of the selective declines in M-HBsAg and L-HBsAg, which precede HBsAg loss during NUC therapy of HBeAg-positive HBV infection,^[30] it did not include M-HBsAg in the HBeAg-negative patients in the REP 301 and REP 401 studies, and occurred in the absence of NUCs and before pegIFN therapy in the REP 301 study.

Recent studies have demonstrated the inactivation/degradation of cccDNA with NUCs^[45,46] and pegIFN,^[47–49] both of which are components of the NAP-based therapies evaluated in this study. These effects may contribute to the more efficient clearance of L-HBsAg through inactivation/clearance of cccDNA. The more effective clearance of L-HBsAg in patients

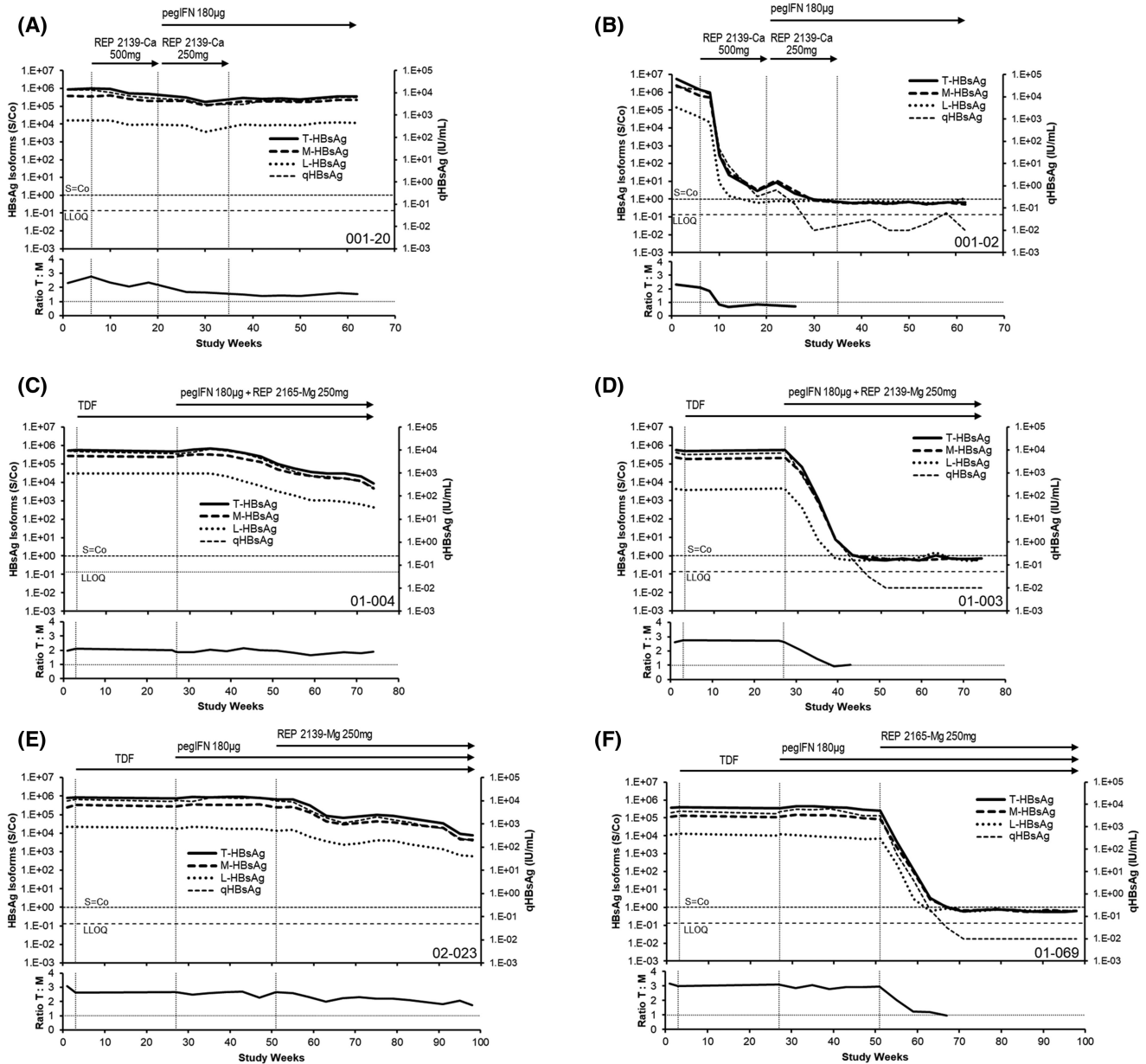


FIGURE 6 Selective decline of HBsAg (S-HBsAg) during NAP-based combination therapy. Representative examples of nonselective (A,C,E) and selective (B,D,F) S-HBsAg response in the REP 301 and REP 401 studies (see Methods) are provided. Individual T-HBsAg, M-HBsAg, and S-HBsAg and qHBsAg responses are provided at the top of each panel and changes in the T-HBsAg-to-M-HBsAg ratio during therapy are indicated in the bottom of each panel. The qHBsAg data was previously published.^[33,38] The serum sample cutoff (S/Co) is indicated for HBsAg isoform assays, and LLOQ (0.05 IU/ml) is indicated for the qHBsAg assay

TABLE 2 Correlation between selective S-HBsAg clearance during therapy and qHBsAg response

qHBsAg response during therapy (decline from baseline)	S-HBsAg decline		p value ^a
	Total		
<2 log ₁₀ IU/ml	10	1	<0.01
>2 log ₁₀ IU/ml	42	39	

^aDetermined by χ^2 analysis.

co-infected with HBV/HDV may reflect lower cccDNA levels in co-infection,^[50] with subsequent clearance of T-HBsAg and M-HBsAg (concomitant with

transaminase flares), suggesting removal of integrated HBV DNA. These open questions highlight the need for additional studies, which should preferably include quantitative HBsAg isoform assays.

The selective S-HBsAg isoform response observed in participants experiencing strong qHBsAg declines validates the selective effects of NAPs in inhibiting the assembly and secretion of spherical SVPs in humans receiving NAP-based therapy. Additional isoform analysis in future studies with NAPs will be useful to examine the antiviral effects of NAPs in humans in more detail.

CONFLICT OF INTEREST

MB and AV are employees of, shareholders in, and inventors of patents assigned to Replicor Inc. MA, JG, VH, MK, and GC are employees of and shareholders in Abbott Diagnostics Inc.

AUTHOR CONTRIBUTIONS

REP 401 study design: Andrew Vaillant and Michel Bazinet. *Patient data collection:* Victor Pântea, Gheorghe Placinta, Iurie Moscalu, Valentin Cebotarescu, Lilia Cojuhari, Pavlina Jimbei, Liviu Iarovoi, Valentina Smesnoi, Tatina Musteata, and Alina Jucov. *Experimental HBV testing:* Mark Anderson, Jeff Gersch, Vera Holzmayer, Mary Kuhns, and Gavin Cloherty. *HBV testing supervision:* Ulf Dittmer. *Data analysis:* Andrew Vaillant and Mark Anderson. *Manuscript draft:* Andrew Vaillant with assistance from all authors.

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SUPPORTING INFORMATION

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