HEPATOLOGY

HEPATOLOGY, VOL. 73, NO. 1, 2021

Rapid Turnover of Hepatitis B Virus Covalently Closed Circular DNA Indicated by Monitoring Emergence and Reversion of Signature-Mutation in Treated Chronic Hepatitis B Patients

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BACKGROUND AND AIMS: Hepatitis B virus (HBV) covalently closed circular DNA (cccDNA) plays a pivotal role in the establishment and persistence of HBV infection. Understanding the turnover time of preexisting cccDNA pools would be helpful in designing strategies to clear HBV by fully blocking the *de novo* generation of cccDNA.

APPROACH AND RESULTS: In this study, we retrospectively monitored the emergence and reversion of the rtM204I/V mutant, a signature lamivudine resistance (LAM^R) mutation serving as a biomarker of cccDNA turnover in liver biopsies and longitudinal serum samples from two clinical trials. Methodologies were optimized to differentially isolate and sequence HBV virion DNA, cccDNA, and HBV RNA from clinical samples. A strong correlation was observed between LAM^R composition of cccDNA with that of serum and intrahepatic HBV RNA in paired liver and serum samples (r = 0.96 and 0.90, respectively), suggesting that serum HBV RNA can serve as a surrogate marker of cccDNA genetic composition when liver biopsies are unavailable. LAM^R mutations emerged and increased from undetectable to 40%-90% within 16-28 weeks in serum HBV RNA from telbivudine-treated patients experiencing virological breakthrough. Similarly, in lamivudine-resistant patients who switched to interferon therapy, serum HBV-RNA population bearing 100% LAM^R mutations fully reversed back to wild type within 24-48 weeks.

CONCLUSIONS: The genetic composition dynamics of serum HBV RNA and biopsy cccDNA in treated HBV patients indicates that cccDNA turnover occurs relatively rapidly (several months), offering a possibility of HBV cure with finite therapy through completely blocking cccDNA replenishment. (Hepatology 2021;73:41-52).

Hepatitis B virus (HBV) infection is a major global public health problem. The virus infects the liver and can cause both acute and chronic infection. An estimated 257 million people are living with HBV, and it contributes to >780,000 deaths every year globally.[1-3] Persistence of HBV infection is attributable to the reservoir of covalently closed circular HBV DNA (cccDNA) within the nuclei of infected hepatocytes, which serves as the template for viral transcription and subsequent

Abbreviations: ALT, alanine aminotransferase; cccDNA, covalently closed circular DNA; CHB, chronic hepatitis B; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; LAM^R, lamivudine resistance; LdT, telbivudine; Nucs, nucleos(t)ide analogues; PegIFN, pegylated interferon alfa-2a; rcDNA, relaxed circular DNA; SDS, sodium dodecyl sulfate; VB, virological breakthrough; WT, wild type.

Received September 20, 2019; accepted March 10, 2020.

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.31240/suppinfo.

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Supported by the funding from Assembly Biosciences, NIH (AI110762, AI123271, and AI134818 to H.G.), National Science and Technology Major Project (2017ZX10202202 to J.S. and 2018ZX10301202 to J.H.), Natural Science Foundation of China (81871668 to J.S.; 81600475 and U1401226 to B.Z.), Guangzhou Science and Technology Plan Project (201804020001 to J.S.), Local Innovative and Research Teams Project of Guangdong Pearl River Talents Program (2017BT01S131), and Guangdong Natural Science and Technology (2016A030313550 to J.S.).

Clinical trial number: NCT00962533, ISRCTN79659320.

replication.[4,5] Clearance of cccDNA is an undisputed goal for the cure of hepatitis B. Because of the inability of all available treatments to eliminate cccDNA from HBV-infected hepatocytes, lifelong therapies are often required and very few patients maintained a sustained viral and clinical remission off-therapy.

Understanding the half-life of cccDNA pools is important to the design of future viral-suppressive strategies for chronic hepatitis B (CHB). The estimated cccDNA copy number ranges widely from 1 to ≥50 per infected hepatocyte.[6,7] Current knowledge of cccDNA decay in infected cells was originally derived from cell-culture and animal studies. The half-life of HBV cccDNA was estimated to be 9 days in confluent cells,[8,9] but ranges from 35 to 57 and 33 to 50 days for duck HBV and woodchuck hepatitis virus (WHV), respectively.[10,11] Some studies took advantage of nucleos(t)ide analogue (Nuc)-based therapies to investigate the decline of cccDNA levels in patients with chronic HBV infection (CHB), and the observed cccDNA decay kinetics led to projections inferring that it may take 10-20 years to clear intrahepatic cccDNA in CHB patients, if not longer.[12-14] However, considering the growing evidence that residual levels of HBV replication and cccDNA replenishment likely continue in patients on long-term Nuc-based therapies,[13,15,16] these earlier cccDNA decay analyses need to be interpreted with caution, especially when qPCR-based cccDNA detection methods were used, given that these early approaches are prone to false-positive amplification of relaxed circular DNA (rcDNA).

One way to study the kinetics of cccDNA turnover resides in the monitoring of its genetic composition over time. Previous studies on the selection and emergence of lamivudine resistance (LAM^R) mutants suggested that HBV genome variability leads to a complex pool of viral quasispecies and mutants archiving in cccDNA.[17] It has been observed that the detection of LAM^R mutations in serum HBV DNA can arise within only months with first-generation Nucs, such as lamivudine and telbivudine (LdT).[18-21] However, the kinetics of LAM^R species emergence in the intrahepatic cccDNA pool remains unclear because of the lack of longitudinal biopsy samples from CHB patients who experienced virological breakthrough (VB), together with the substantial technical challenges associated with the study of intrahepatic cccDNA. Nevertheless, one could envision getting a "snapshot" of cccDNA pool

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DOI 10.1002/hep.31240

Potential conflict of interest: *Dr. Mercier was employed by Assembly Biosciences. Dr. Cai is employed by Assembly Biosciences. Dr. Colonno is employed by and owns stock in Assembly Biosciences. Dr. Guo received grants from and owns stock in Arbutus. He consults for Assembly and Aligos. Dr. Hou consults for and received grants from Bristol-Myers Squibb and Johnson & Johnson. He consults for AbbVie, Arbutus, Gilead, and Roche. Dr. Huang is employed by and owns stock in Assembly Biosciences. Dr. Zong was employed by Assembly Biosciences.*

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through surrogate genetic markers by following the emergence of cccDNA LAM^R mutations in patients who experience VB. A promising surrogate marker for cccDNA in this context is serum HBV RNA, though its biogenesis and biological function in the HBV life cycle await further investigation.[22] Recent studies have shown the association between serum HBV RNA and viral persistence as well as clinical response to therapies. Furthermore, experiments conducted in HBV-infected humanized mice demonstrated that the detection of serum HBV RNA is reflective of cccDNA activity.[23-26]

In this study, we used the lamivudine signature resistance substitution, rtM204I/V (LAM^R), as a signature biomarker to assess the turnover of cccDNA molecules in CHB patients. Longitudinal clinical samples, including sera and liver biopsies from two randomized, controlled clinical trials, were retrospectively analyzed to monitor the incidence of LAM^R mutations in serum HBV DNA and RNA, as well as in intrahepatic HBV DNA/RNA and cccDNA. Our research goal was to confirm whether serum HBV RNA can serve as a surrogate genetic marker for cccDNA and then calculate the half-life of cccDNA turnover based on the analyses of serum HBV RNA.

Materials and Methods

PATIENT SAMPLES

This was a retrospective analysis of patients from two phase IV clinical trials. The EFFORT (EFFicacy Optimization of Response to Telbivudine) study (registration number: NCT00962533) is a randomized study to evaluate the efficacy and safety of the Roadmap strategy.[27,28] Six hundred six nucleos(t)ide-naïve, hepatitis B e antigen (HBeAg)-positive CHB patients were randomized into the OPTIMIZE group or MONO group between 2009 and 2012. Patients in the OPTIMIZE group started with LdT treatment at baseline, and adefovir was added to patients with suboptimal response (HBV DNA ≥300 copies/mL at 24 weeks) from weeks 28 to 104; patients with early virological response (HBV DNA <300 copies/mL at week 24) continued LdT monotherapy until week 104. Patients in the MONO group received LdT monotherapy from baseline until week 104. All patients with LdT monotherapy were added on adefovir as rescue therapy once VB was developed. All patients underwent liver biopsies at baseline and at week 104. To exclude the potential impact of adefovir on the evolution of drug resistance mutation, only those patients from the MONO group who developed the signature rtM204I/V mutation during LdT monotherapy were included in this study.

Study ML18376 (registration number: ISRCTN79659320) is a randomized, open-label study aiming to explore the effect of pegylated interferon alfa-2a (PegIFN) on the rescue treatment of patients with LAM^R.[29] This study enrolled 235 HBeAg-positive CHB patients with LAM^R from 2005 to 2008. Patients were randomized (2:1) to PegIFN for 48 weeks with 24 weeks' follow-up or adefovir alone for 72 weeks. Patients in both groups continued LAM treatment for the first 12 weeks. Only patients from Nanfang Hospital were selected for further analysis in this study. Detailed information about the EFFORT study and ML18376 study have been published.[27,29] This study was conducted in compliance with the ethics principles of the Declaration of Helsinki and Good Clinical Practice and China regulatory requirements. The two registered clinical trials have been approved by ethics committee of Nanfang Hospital. Written informed consent was obtained from all patients.

EXTRACTION, AMPLIFICATION, AND QUANTITATION OF SERUM HBV DNA/RNA

Serum HBV DNA/RNA were copurified with QIAamp MinElute Virus kit (Qiagen, Hilden, Germany) from 200 μL of patient serum. Eluted DNA/RNA mixture was subjected to PCR (Quantabio, Beverly, MA) for DNA analysis by a pair of pan-genotype primers covering the reverse transcriptase region, RT_s: 5′-CTGCTGGTGGCTCCAGTT-3′ - and RT_as: 5′-GCCTTGTAAGTTGGCGAGAA-3′-. HBV RNA was purified following a subsequent digestion with 1 U of DNase I (Thermo Fisher Scientific, Waltham, MA) for 30 minutes at 37°C to eliminate HBV-DNA contamination. To ensure that no residual HBV DNA existed after DNase I digestion, all the DNase I–treated samples were confirmed to be negative by subsequent PCR analysis to prove that residual serum DNA was eliminated completely (Supporting Fig. S1). The reverse-transcriptase region of serum HBV RNA was amplified by RT-PCR using the qScript XLT one-step RT-PCR kit (Quantabio). HBV DNA in plasma samples were quantified by the Roche COBAS TaqMan HBV Test. Serum HBV RNA was

quantified by one-step reverse-transcription RT-qPCR in a LightCycler 480 Instrument II system (Roche, Mannheim, Germany) with the TaqMan probe method as described.[30]

PURIFICATION OF INTRAHEPATIC cccDNA AND HBV DNA/RNA AND AMPLIFICATION OF REVERSE-TRANSCRIPTASE REGION

Snap-frozen liver biopsies were digested with 0.5% (w/v) collagenase A (Sigma-Aldrich, St. Louis, MO) at 37°C for 30 minutes in the presence of 5 mM of $CaCl_2$ and 1 mM of dithiothreitol for optimal dissociation to single hepatocytes. HBV cccDNA was extracted by a modified Hirt method.[8,9]. Briefly, the hepatocyte nuclei were lysed with 0.6% (w/v) SDS (sodium dodecyl sulfate), followed by the addition of 1.2 M of CsCl and 0.4 M of KAc to form a protein-SDS precipitation complex. The mixture was centrifuged, and the Hirt supernatant was loaded onto the QIAquick Spin column (Qiagen, Hilden, Germany) and washed twice with PE buffer before elution of Hirt DNA (protein-free or deproteinated DNA) with nuclease-free water. Hirt DNA was then digested with T5 exonuclease (New England Biolabs, Ipswich, MA) at 37°C for 30 minutes to remove protein-free rcDNA contaminants. The cccDNA was subjected to PCR amplification of the reverse-transcriptase region for DNA sequence analysis. The pelleted protein-SDS precipitation complex and the column flow-through were combined for isolating HBV RNA and core DNA extraction, as described above for serum HBV-DNA/RNA extraction.

PERCENTAGE COMPOSITION OF rtM204I/V IN HBV cccDNA, VIRAL DNA, AND RNA

PCR and RT-PCR products of the reverse-transcriptase region were purified by the QIAquick PCR purification kit (Qiagen, Hilden, Germany) and subjected to population sequencing with an HBV pan-genotypic primer, RT seq 5′-GTGTTAC AGGCGGGGTTTTTCTTG-3′. Percentages of rtM204I/V mutations were calculated using Sequencher software (Gene Codes, Ann Arbor, MI).

STATISTICAL ANALYSIS

Coefficients (r^2) and *P* values (F-test) in correlation analyses were determined with GraphPad Prism software (version 8.0; GraphPad Software Inc., San Diego, CA), using the linear regression model. cccDNA half-life for each patient was inferred using the first-order decay equation $N(t) = N_0 e^{-\lambda t}$,[31] specifically its equivalent formula $N(t) = N_0(0.5)^{t/t_{½}}$, where N(t) is the remaining quantity at time t, N_0 the initial quantity, and $t_{½}$ the half-life.

Results

PATIENT CHARACTERISTICS

Five patients treated with LdT monotherapy from the EFFORT study and 9 patients from the ML18376 study were included in this evaluation. All patients were HBeAg positive at baseline. The flowchart of patient selection process is illustrated in Fig. 1.

In brief, for the EFFORT study, 299 patients were included in the MONO group and 77 of them developed VB as well as the rtM204I/V mutation. These patients with emerging resistance received adefovir as rescue therapy. To minimize the potential impact of adefovir on the evolution of mutational resistance patterns, only patients who developed resistance between weeks 88 and 104 were included. In addition, to facilitate HBV-DNA/RNA sequencing in serum samples, we only selected the patients with HBV DNA >500 IU/mL at every time point before VB. Based on the availability of snap-frozen liver biopsy samples at baseline and at week 104, 5 patients were included for final analysis.

For the ML18376 study, only 4 patients from the adefovir group had snap-frozen liver biopsy samples at baseline and were included for analysis of the correlation between cccDNA composition and intrahepatic/serum HBV DNA and RNA. In addition, 5 patients with sustained HBeAg-positive and HBV DNA >3 $\log_{10}$ IU/mL until the end of the follow-up period, who presented 100% rtM204I/V mutation at baseline, were also included for kinetics analysis of rtM204I/V in serum HBV DNA/RNA during the interferon rescue period.

VALIDATION OF METHODOLOGIES THAT SELECTIVELY ISOLATE INTRAHEPATIC HBV cccDNA, DNA, AND RNA FROM LIVER BIOPSIES

An updated version of cccDNA isolation methods has been recently developed for cell-culture systems,[32] which requires the separation of cccDNA from nuclear genomic DNA and cytoplasmic rcDNA by a modified Hirt method, followed by T5 exonuclease digestion to remove the contaminating rcDNA. We have further optimized this method for isolation of intrahepatic HBV cccDNA, DNA, and RNA from snap-frozen liver biopsies (Materials and Methods). Experimental flowcharts for the analysis of HBV cccDNA, DNA, and RNA from CHB biopsies and sera (exclusive of cccDNA) are shown in Supporting Fig. S2A,B, respectively. Population sequencing was then performed on these materials following (RT)-PCR reactions. The efficient removal of contaminating rcDNA from biopsy extracts was validated with genetically distinct spiked-in rcDNA (Supporting Material and Supporting Fig. S3).

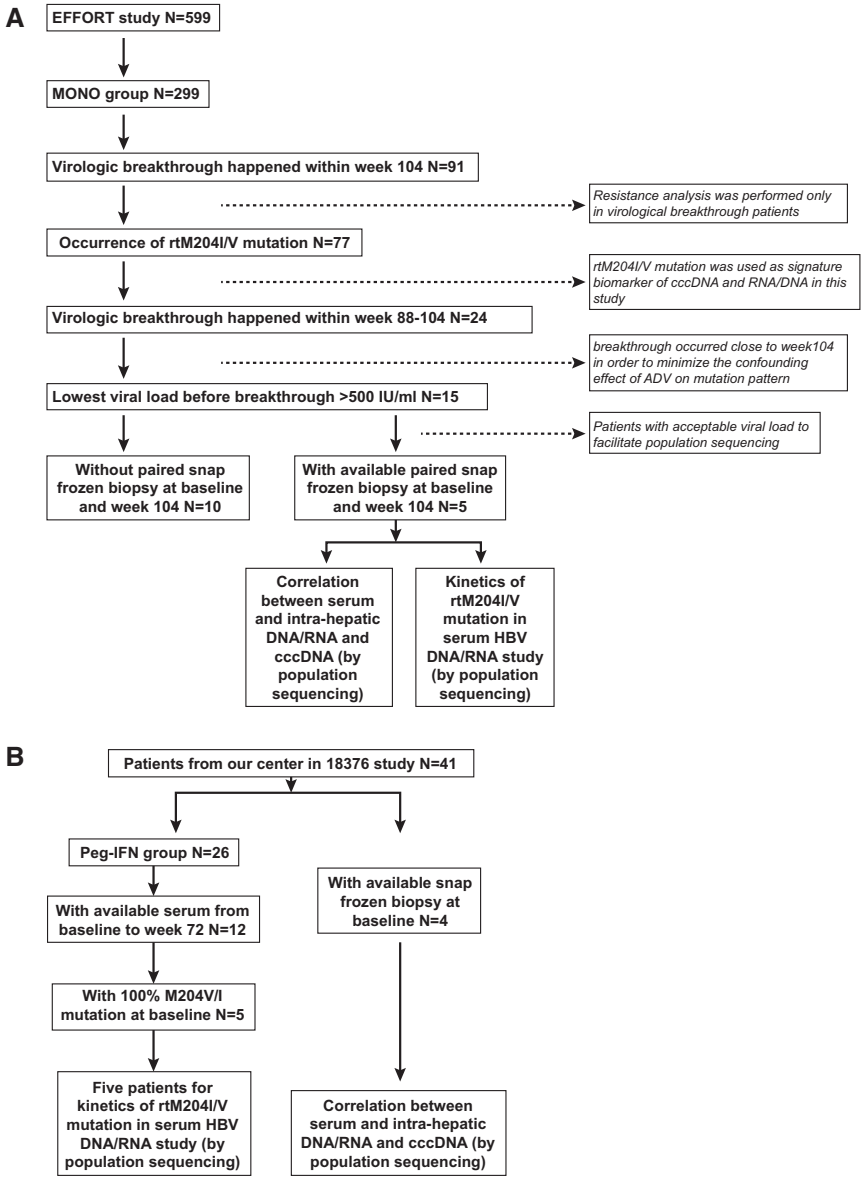

FIG. 1. Flowchart of patient selection. Abbreviation: ADV, adefovir dipivoxil.

The result showed that the optimized T5 exonuclease digestion can efficiently exclude an excess (40-fold) of LAM^R rtM204V rcDNA that was spiked in a Hirt DNA extract containing WT cccDNA. As for CHB serum samples, viral DNA/RNA were coextracted first, followed by viral DNA analysis immediately, then HBV RNA analyses, including population sequencing, were performed upon removal of DNA content by DNase I treatment. These methodologies allowed us to closely monitor the emergence of LAM^R variants in cccDNA, HBV DNA, and RNA from paired CHB liver biopsies and serum samples, a powerful tool kit to study HBV genetic composition and the kinetics of cccDNA turnover.

SEQUENCE CORRELATIONS BETWEEN SERUM AND INTRAHEPATIC HBV DNA/RNA AND cccDNA

All the 5 patients in the EFFORT study had liver biopsy and blood sampling on the same day, whereas the time of liver biopsy was earlier than that of blood sampling for the 4 patients from the study, ML18376, which was 8 days (patient 3), 13 days (patient 29), 27 days (patient 16), and 56 days (patient 47) earlier. We found a significant difference in the LAM^R ratio between serum RNA and liver biopsy cccDNA in samples with an interval of 27 and 56 days (Supporting Fig. S4). Therefore, we chose seven samples (5 patients from the EFFORT study and 2 patients from the ML18376 study) with an interval of <2 weeks for further correlation analysis.

Population sequencing of serum HBV DNA confirmed that all of the 9 patients bore 100% rtM204I/V LAM^R mutation at week 104 of the EFFORT study or at baseline of the ML18376 study (Fig. 2 and Supporting Fig. S4). Remarkably, a significant percentage of rtM204I/V LAM^R mutant (40%-90%) was found in serum HBV RNA, intrahepatic viral RNA, and cccDNA at the corresponding time points in biopsy samples in all 7 patients (Fig. 2A). Figure 2B shows the tight correlation between LAM^R composition of both serum HBV RNA and intrahepatic HBV RNA ($r^2 = 0.95$; $P < 0.01$). Importantly, the genetic compositions of both RNA species are highly correlated with that of cccDNA, with r^2 coefficients of 0.90 and 0.96 ($P < 0.01$), respectively (Fig. 2C,D). In contrast, the composition of intrahepatic HBV DNA is not correlated with that of cccDNA (Fig. 2E). Collectively, the data from paired serum and liver biopsies demonstrated that the serum HBV-RNA LAM^R composition correlates well with intrahepatic RNA and cccDNA, hence validating that serum HBV RNA can reflect the genetic composition of cccDNA.

RAPID EMERGENCE OF LAM^R MUTATIONS IN HBV RNA DURING VB IN THE EFFORT STUDY

The establishment of a strong correlation between serum HBV RNA and intrahepatic cccDNA enables the analysis of cccDNA turnover kinetics by monitoring the composition of HBV RNA in longitudinal serum samples. Samples from 5 HBeAg-positive, LdT-resistant CHB patients from the EFFORT study (patients 7, 17, 20, 26, and 31) were analyzed to determine the kinetics of cccDNA turnover during VB using serum HBV RNA as a cccDNA genetic biomarker. Population sequencing revealed that LAM^R variant rtM204I/V in serum DNA emerged between weeks 64 and 88 in all 5 patients evaluated. The emergence of serum LAM^R HBV-RNA variants was generally delayed compared to the detection of serum LAM^R DNA, but ultimately accumulated to >40%-90% of total serum HBV RNA by week 104 (Fig. 3). It took 16-28 weeks for the emergence of LAM^R in serum HBV RNA, which subsequently became enriched by the end of treatment (Table 1). The inferred half-life of cccDNA turnover from these results ranged from 6.9 to 21.7 weeks. These results suggested a rapid replenishment of the wild-type (WT) cccDNA pool with LAM^R cccDNA. Alanine aminotransferase (ALT) levels fluctuated moderately during treatment in some patients (Fig. 3), but did not appear to impact the composition or rate of HBV-RNA turnover.

REVERSION OF LAM^R MUTATIONS BACK TO WT IN PATIENTS WHO SWITCHED FROM LAM TO PegIFN THERAPY

HBV DNA and HBV RNA from longitudinal serum samples of 5 patients nonresponsive to PegIFN (patients 13, 46, 50, 53, and 194), defined by sustained HBeAg-positive and sustained HBV DNA >3 log IU mL, were RT-PCR amplified and sequenced. Population sequencing of serum DNA and HBV RNA from all 5

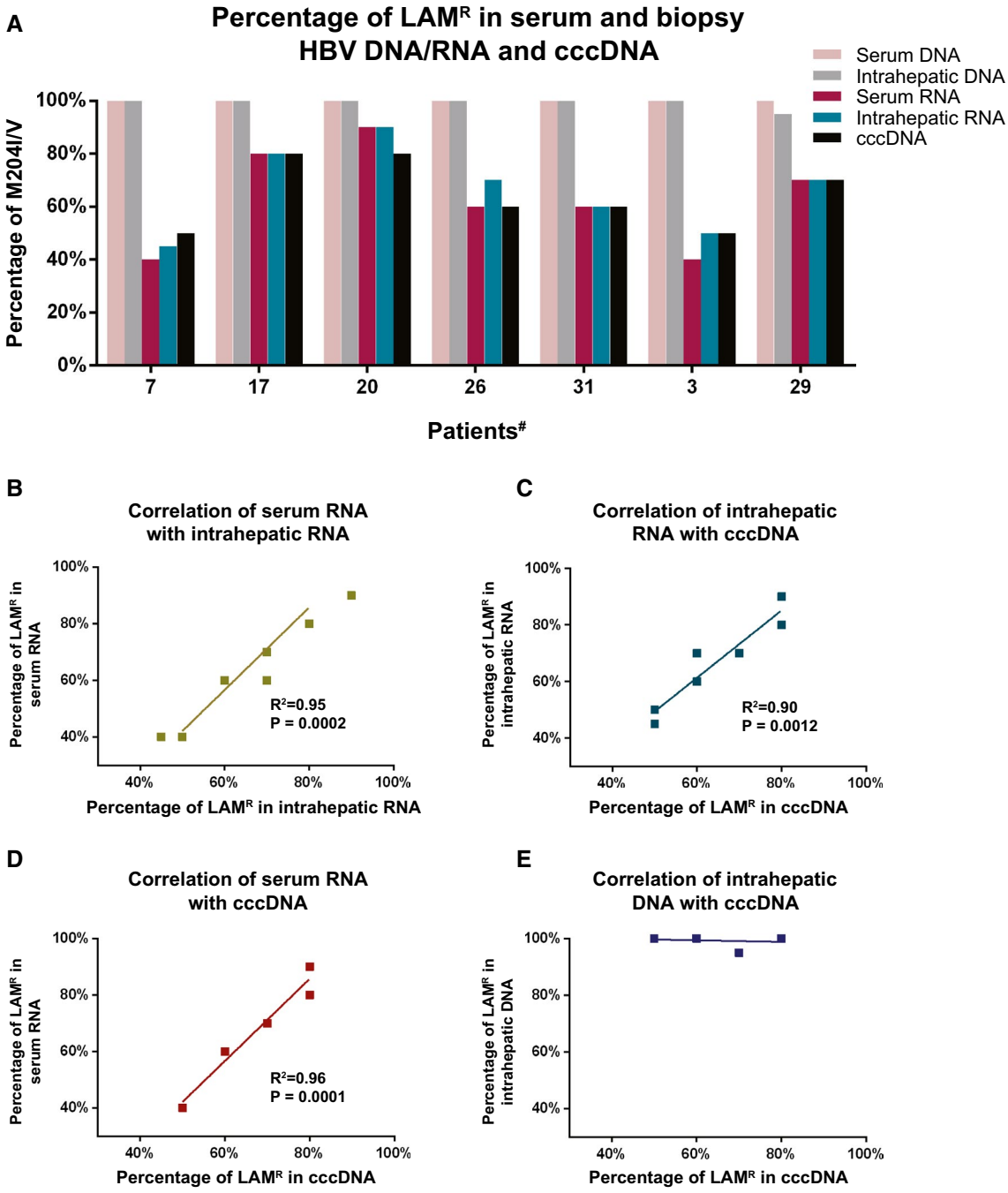

FIG. 2. Genetic composition of HBV cccDNA, DNA, and RNA. Percentage of LAM^R rtM204I/V mutation in serum and liver biopsy samples of 5 EFFORT patients collected at week 104 and 2 ML18376 patients collected at baseline (A). The correlation of LAM^R composition among intrahepatic RNA, intrahepatic DNA, cccDNA, and serum HBV RNA were analyzed (B-E).

patients showed the presence of 100% LAM^R at baseline (Fig. 4). Furthermore, a reversion to WT rtM204 was observed in both serum HBV DNA and HBV RNA by week 72. Remarkably, in spite of limited sample availability in terms of time points (only four time points with 24 weeks apart), the data showed that the full conversion of HBV RNA from LAM^R to WT was completed in 24-48 weeks (Fig. 4). Considering that HBV mutant in all patients converted to 100% WT in that time frame and that the detection limit of our

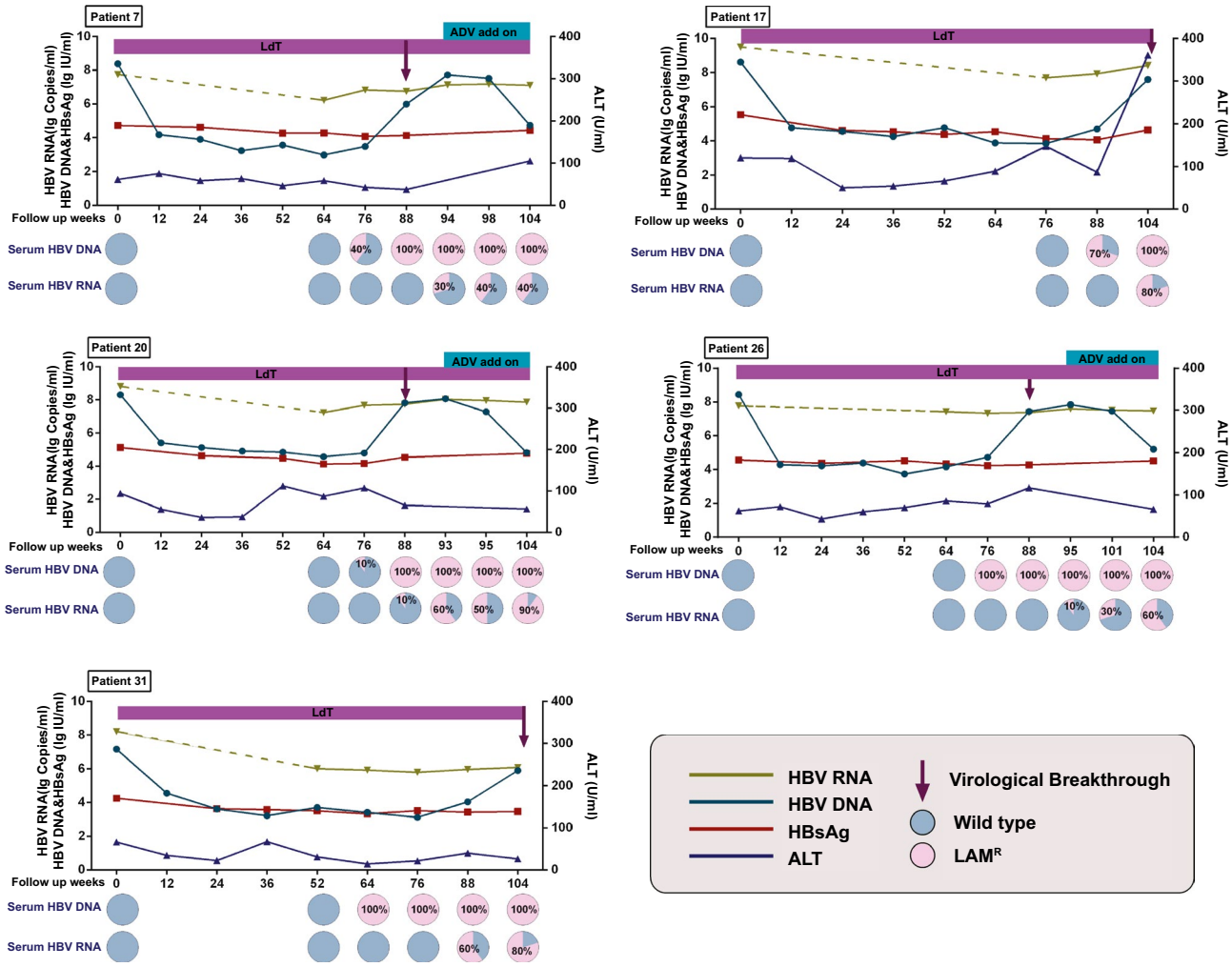

FIG. 3. Dynamic change of LAMR in serum HBV RNA and DNA during the development of VB in the EFFORT study (patients 7, 17, 20, 26, and 31). Abbreviations: ADV, adefovir dipivoxil; HBsAg, hepatitis B surface antigen; Ig, immunoglobulin; LdT, telbivudine.

population sequencing methodology was determined to be 2.5% (Supporting Material and Supporting Fig. S5), this result suggested that the cccDNA turnover rate was conservatively estimated to range between 5.6 and 11.1 weeks, using 2.5% as the threshold of residual LAM^R composition (Table 1). Such serum HBV-RNA/cccDNA turnover kinetics are similar to results obtained in the EFFORT cohort and show a consistent rate of biosynthesis and replenishment of the cccDNA pool across CHB patients from both studies. The modest fluctuations of ALT levels during treatment in ML18376 patients did not appear to impact the composition or rate of HBV-RNA turnover, which is similar to the case for the EFFORT patients.

Discussion

In this study, we have developed an innovative and sensitive way to study HBV cccDNA turnover by monitoring the emergence and reversion of LAM^R signature mutations in treated CHB patients. The tight correlation between the genetic composition of serum HBV RNA and intrahepatic cccDNA was initially proved by evaluating paired liver biopsy and serum samples (Fig. 2). Based on the kinetics of LAM^R mutations in serum HBV RNA in longitudinal samples, we estimate that cccDNA turnover occurs within several months, which is significantly shorter than previously estimated (decades).

TABLE 1. Kinetics of cccDNA Turnover Inferred From Changes in Serum pgRNA Genetic Composition

Patient No.	Maximal cccDNA Turnover at EOT*	Time to Maximal Turnover (Weeks)†	Inferred Half-Life (weeks)‡
7	40%	16	21.7
17	80%	16	6.9
20	90%	28	8.4
26	60%	16	12.1
31	80%	28	12.1
13	100%	<48	<11.1
45	100%	<48	<11.1
50	100%	<24	<5.6
53	100%	<48	<11.1
194	100%	<48	<11.1

*Change in serum pgRNA Nuc^R composition by population sequencing.
†Shortest time elapsed from 0% Nuc^R (EFFORT) or 0% WT (ML18376) to percentage composition reached at EOT.
‡Based on the assumption that cccDNA decay is subject to exponential decay. For the ML18376 study, a theoretical residual Nuc^R composition of 2.5% was used to reflect the resolution of the population sequencing methodology.
Abbreviations: EOT, end of treatment; Nuc^R, NUC resistance; pgRNA, pregenomic RNA.

Our limited knowledge about the turnover rate of cccDNA mainly originated from cell-culture and animal models. *In vitro* studies of cccDNA stability have demonstrated that the half-life of HBV cccDNA is <10 days in confluent cells, whereas *in vivo* studies of nonhuman hepadnaviruses in infected animals estimated the half-life of cccDNA to be of several weeks.[8-11] The limited data about the decay of cccDNA in CHB patients with long-term Nuc treatment led to the predictions that it may take decades to completely clear cccDNA.[12-14] However, it has been also suggested that the intrahepatic HBV-DNA replication and replenishment of cccDNA pools remain persistent during prolonged Nuc treatment, even though serum HBV-DNA levels become undetectable by qPCR.[13,15] Hence, the half-life of cccDNA might have been overestimated based on the cccDNA decay kinetics in Nuc-treated patients and thus needs to be revisited.

In this study, LAM^R mutations were utilized as a genetic biomarker to differentiate preexisting from synthesized viral nucleic acids (HBV DNA, RNA, and cccDNA) in paired serum and liver biopsy samples from CHB patients with VB during LdT treatment. The selection of rtM204I/V as the signature biomarker was based on several reasons: First, rtM204I/V is a high-frequency LAM^R mutation site, especially in patients with LAM or LdT antiviral therapy. Second, the development or reversion of the rtM204I/V mutant in serum can occur rapidly (within several months) with continuation or withdrawal of antiviral therapy. This provides an opportunity to observe the dynamic change of LAM^R in a relatively short time window. Last, patients with rtM204I/V develop VB with relatively high viral loads, which also facilitate the amplification and sequencing of LAM^R in serum samples.

Through paired liver biopsy and serum samples, we found that the percentage composition of LAM^R mutations in serum HBV RNA correlated well with that of intrahepatic RNA and cccDNA, but not with intrahepatic DNA, demonstrating that serum HBV RNA is a reliable surrogate marker of cccDNA genetic composition in clinical settings. It is worth noting that, in samples with a time interval >2 weeks between the date of liver biopsy and blood sampling, the correlation between serum HBV RNA and cccDNA decreased, further supporting a rapid and dynamic turnover of cccDNA and HBV DNA/RNA during prolonged antiviral therapy.

By using the LAM^R composition of serum HBV RNA as a genetic marker of cccDNA, we were able to study the kinetics of cccDNA turnover in LAM^R CHB patients from two distinct clinical studies. The appearance of LAM^R in serum HBV RNA suggests a cccDNA half-life of 6.9-21.7 and 5.6-11.1 weeks for patients in the EFFORT and ML18376 studies, respectively. Remarkably, several patients were able to reach a genetic turnover ranging from 95% to 100% within 16-48 weeks. Importantly, the relatively rapid decay of intrahepatic cccDNA pools observed in these patients suggests that the cccDNA reservoirs are more dynamic than previously thought. Based on our data, a model for cccDNA turnover has been proposed in Fig. 5, which shows that the full turnover of WT cccDNA populations occurs in several months. Theoretically, the LAM^R mutations found in both cccDNA and HBV-RNA populations originate from the replicating rcDNA. Under the selective pressure by NUC treatment, any preexisting minor population of LAM^R mutant is selected and accumulates over time by recycling the mutant rcDNA into the nucleus to form cccDNA or *de novo* infection of either the new cell or the originally infected cell. Subsequently, the LAM^R mutations enriched in the cccDNA population are reflected in the HBV-RNA population after cccDNA transcription.

The underlying mechanism(s) for cccDNA clearance in hepatocytes remains unclear. Earlier studies

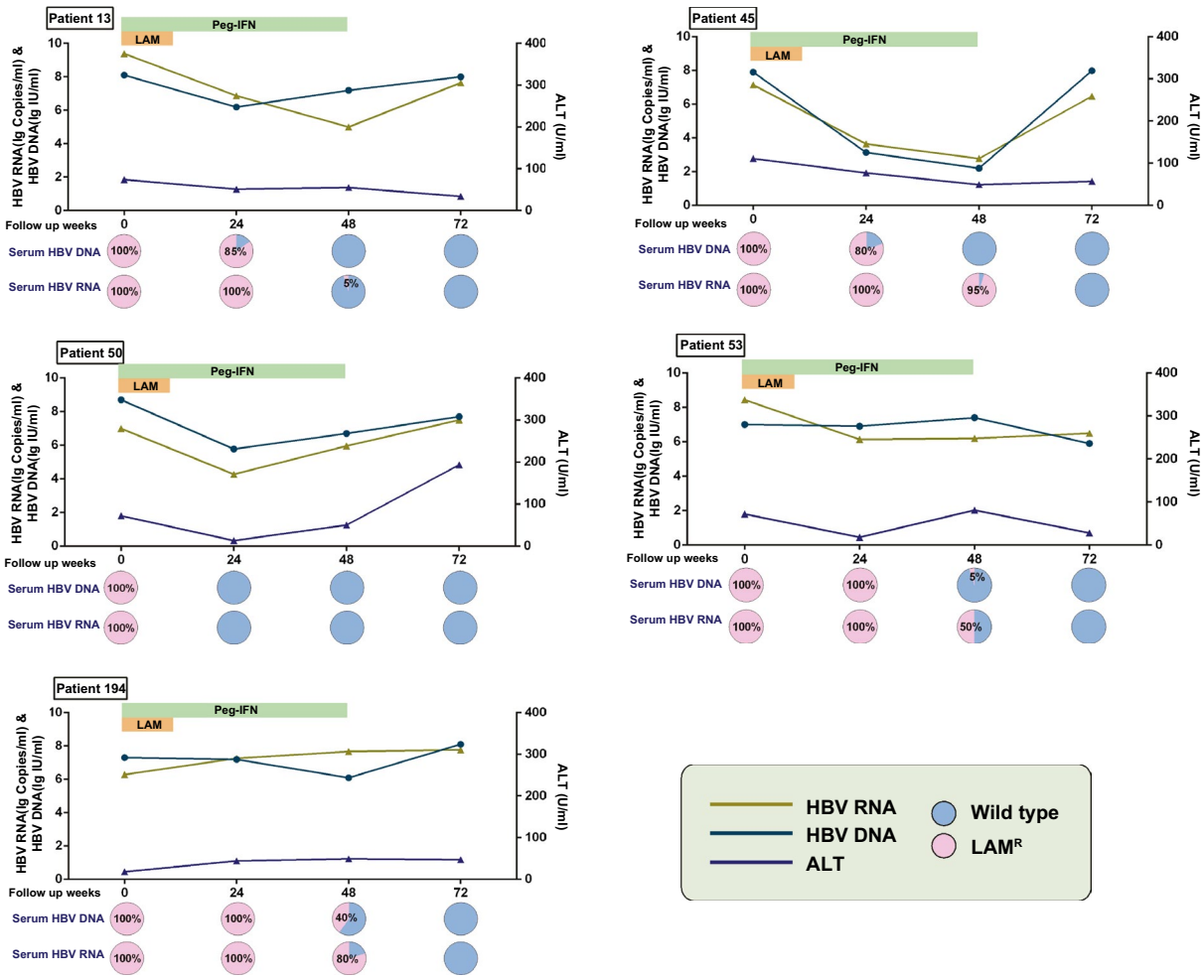

FIG. 4. Dynamic change of LAM^R in serum HBV RNA and DNA during the reversion of LAM^R in ML18376 study (patients 13, 45, 50, 53, and 194). Abbreviation: Ig, immunoglobulin.

in HBV-infected chimpanzees demonstrated that cccDNA clearance can occur in acutely infected livers without cell destruction.[33-35] Other studies in the woodchuck model showed that the killing of hepatocytes may play a role in inducing hepatocyte proliferation, which may also contribute to cccDNA loss.[36-38] However, another study demonstrated that the amount of hepatocyte turnover during resolution of transient WHV infections was lower when virus replication was inhibited with entecavir, indicating that mechanisms other than hepatocyte death were responsible for elimination of cccDNA during recovery from transient infections.[39] Patients retrospectively selected for the current study did experience slightly elevated ALT levels during VB (Fig. 3), which coincided with the observed cccDNA population turnover. It is unclear at this time whether these low levels of indiscriminate cell death played any significant role in the rapid replacement of WT cccDNA by LAM^R cccDNA observed during VB in patients in the EFFORT study.

Because of the limited number and size of liver biopsy samples available to this retrospective study, our main goal was to assess whether serum HBV RNA could qualitatively reflect cccDNA in terms of genetic composition, but not to quantify their absolute copy numbers. This "proof-of-concept" study conceptually and technically established an approach to predict the turnover of cccDNA by monitoring the dynamics of cccDNA genetic markers in CHB patients. The direct measurements of cccDNA dynamics will be included in larger cohorts of future prospective studies.

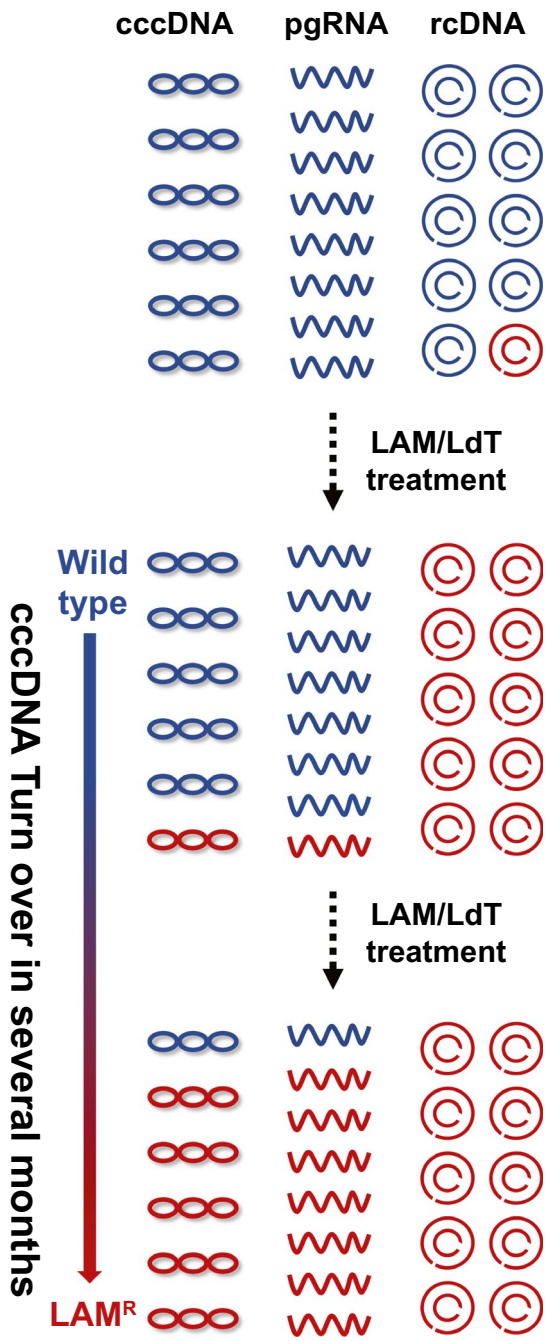

FIG. 5. cccDNA turnover model. In the proposed model, the genetic composition of serum HBV RNA, a genetic biomarker for cccDNA turnover, changes within several months, which is much faster than previously thought. Abbreviation: pgRNA, pregenomic RNA.

In summary, our data demonstrate that serum HBV RNA can serve as a reliable surrogate genetic biomarker for cccDNA. These longitudinal studies indicated that the preexisting cccDNA pools might decay faster than previously predicted, with a revised turnover rate of several months, not decades. Understanding the turnover rate of cccDNA pools would clearly aid the design of more-effective antiviral treatment strategies and regimens. To clear cccDNA from infected cells, if direct targeting of cccDNA is not possible, two critical hurdles need to be overcome. First, viral replication and cccDNA replenishment need to be completely blocked, and, second, the pool of preexisting cccDNA must be exhausted in a reasonable time frame. With the advent of potent antivirals, there is a greater possibility in the future that active viral replication and cccDNA replenishment can be completely inhibited. The current results indicate that, under conditions of potent suppression of viral replication, such as the combination of NUC with small interfering RNA or capsid inhibitor, cccDNA clearance may not be impossible someday.

Author Contributions: Q.H., R.C., H.G., J.H., and J.S. were involved in the study design. Q.H., B.Z., D.C., Y.Z., Y.W., S.L., J.H., and J.S. collected data. Q.H., B.Z., D.C., A.M., H.G., R.C., and J.S. analyzed and interpreted the data. Q.H., B.Z., D.C., H.G., R.C., and J.S. wrote the manuscript. All the authors had full access to the final version of the report and agreed to the submission.

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Supporting Information

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