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Sotty Jules[1], Bablon Pierre[1], Lekbaby Bouchra[1], Augustin Jérémy[1,2], Girier-Dufournier Morgane[1], Langlois Lucas[1], Dorival Céline[3], Carrat Fabrice[3], Pol Stanislas[4], Fontaine Hélène[4], Sarica Nazim[5], Neuveut Christine[5], Housset Chantal[1], Kremdsorf Dina[1], Schnuriger Aurélie[1,6]*, Soussan Patrick[1,6] *$.

1: Sorbonne Université, Institut National de la Santé et de la Recherche Médicale (INSERM), Centre de Recherche de Saint Antoine (CRSA), Paris, France;
2: Université Paris-Est Créteil, Département de Pathologie, Hôpital Henri Mondor, Créteil, France;
3: Sorbonne Université, INSERM, Institut Pierre Louis d'Epidémiologie et de Santé Publique, Département de santé publique, Hôpital Saint-Antoine, AP-HP, Paris, France;
4: Université de Paris, AP-HP, Département d'hépatologie, Hôpital Cochin, Paris, France;
5: Institut de Génétique Humaine, Université de Montpellier, Laboratoire de Virologie Moléculaire CNRS-UMR9002, 34000 Montpellier, France.
6: Assistance Publique - Hôpitaux de Paris (AP-HP).Sorbonne Université, Département de Virologie, GHU Paris-Est, Paris, France;

*: Contributed equally

$ Corresponding author: patrick.soussan@inserm.fr

Keywords: Viral hepatitis, HBV, chronic infection, Chronic hepatitis, Viral genome diversity, Viral circulating forms, Alternative splicing, Viral cycle, HBV pregenomic RNA, Liver disease.

Abstract word count: 257
Word count: 4657.
Number of figures: 5

Number of tables: 2

Acknowledgements:

We thank Pr. S. Urban for the kind gift of Hepg2-NTCP cells, Drs. J. Pol and C. Sureau for helpful discussions.

Financial support

This work was supported by funding from Agence Nationale de la Recherche sur le Sida et les Hépatites (ANRS) (grant numbers ECTZ 103985 and ECTZ 163186) and Fondation pour la Recherche Médicale (FRM) (grant number EQU202003010517).

Authors contributions

Conceptualization: SP.

Experiments and procedures: SJ, SA, BP, LB, G-DM, LL, SN, NC, SP.

Data analysis: SJ, SA, KD, SP.

Writing, reviewing and editing: SJ, SA, BP, LB, AJ, G-DM, LL, DC, CF, PS, FH, SN, NC, HC, KD, SP.

Conflict of interest

All authors declare that they have no current financial arrangement or affiliation with any organization that may have a direct influence on their work.

The ANRS CO22 HEPATHER cohort is sponsored and funded by INSERM-ANRS and established in collaboration with Association Française pour l'Etude du

Foie (AFEF). The cohort received supports from Agence Nationale de la Recherche (ANR), Direction Générale de la Santé (DGS), MSD, Janssen, Gilead, Abbvie, BMS, and Roche. The public/private partnership is built in total transparency through a specific contract. The pharmaceutical companies are not involved in scientific decisions. The sponsor did not contribute to the study design and drafting of the study. The sponsor played no role in data collection, data analysis or data interpretation. The other funding sources played no role in study design, data collection, data analysis, data interpretation, or drafting the study.

CF had full access to all data in the cohort study.

Abstract

Background:

Besides the prototypical Hepatitis B virus (HBV) infectious particle, which contains a full-length double-stranded DNA (flDNA), additional circulating virus-like particles, which carry pregenomic RNA (pgRNA), spliced1RNA (sp1RNA) or spliced-derived DNA (defDNA) forms have been described. We aimed to determine the level of these four circulating forms in patients and to evaluate their impact on viral lifecycle.

Methods:

Chronic HBV untreated patients (n=162), included in the Hepather cohort, were investigated. Pangenomic qPCRs were set up to quantify the four circulating forms of HBV nucleic acids (HBV_{naf}). *In vitro* infection assays were performed to address the impact of HBV_{naf}.

Results:

Hierarchical clustering individualized two clusters of HBV_{naf} diversity among patients: i)cluster 1 (C1) showing a predominance of flDNA; ii)cluster 2 (C2) showing various proportions of the different forms. HBeAg-positive chronic hepatitis phase and higher viral load (7.0±6.4 *vs* 6.6±6.2 Log_{10} copies/mL; p<0.001) characterized C2 compared to C1 patients. Among the different HBV_{naf}, pgRNA was more prevalent in C1 patients with high *vs* low HBV viral load (22.1%±2.5% *vs* 4.1%±1.8% of HBV_{naf}, p<0.0001) but remained highly prevalent in C2 patients, whatever the level of replication. C2-patients samples used in infection assays showed that: i)HBV_{naf} secretion was independent of the viral strain; ii)the viral cycle efficiency differed according to the proportion of HBV_{naf} in the inoculum, independently of cccDNA formation. Inoculum enrichment before infection suggests that pgRNA-containing particles drive this impact on viral replication.

Conclusion:

Besides the critical role of HBV replication in circulating HBV_{naf} diversity, our data highlight an impact of this diversity on the dynamics of viral cycle.

Introduction

Hepatitis B virus (HBV) remains a major public health problem, affecting more than 250 million people chronically infected worldwide. Chronic hepatitis related to HBV infection may progress towards development of liver cirrhosis and contribute to the hepatocellular carcinoma (HCC) occurrence [1]. The dynamics of this evolution is determined by both host and virological features. The prototype of HBV (Dane particles) comprises an outer lipid viral envelope surrounding an icosahedral nucleocapsid, which contains a 3.2 kb full-length double-stranded relaxed circular DNA (flDNA). The latter is produced by reverse transcription from the pregenomic RNA (pgRNA) within nucleocapsid in infected hepatocytes [2]. Quantification of Dane particles (flDNA) is routinely performed in clinical practice to assess the level of replication and the efficiency of antiviral therapy [1]. In addition to Dane particles, other viral and subviral particles are released in the bloodstream, including empty particles (the main circulating viral form) or, to a lesser extent, naked free capsids and nucleocapsids [3, 4]. Extracellular vesicles (exosomes), containing HBV genome, have also been described [5].

Besides flDNA, viral particles may contain truncated DNA as well as pregenomic, subgenomic, truncated and spliced viral RNA species. Most virion-like particles containing truncated viral DNA are derived from the spliced pgRNA isoforms after reverse transcription. Due to RNA splicing, incomplete viral genome generates defective particles (dHBV) unable to self-replicate [6-8]. To date, 20 different spliced RNA isoforms (spRNA) have been described, mainly identified as their corresponding truncated DNA forms within circulating dHBV in HBV infected cells [9]. The predominant pgRNA spliced variant (sp1RNA) has one third of the viral genome deleted and it encodes Hepatitis B Spliced-derived Protein (HBSP). The proportion of sp1RNA-

related defective DNA (defDNA) in dHBV particles vary from 0 to 70% of all circulating HBV DNA in chronically infected patients and its presence has been associated with a progression of liver fibrosis and HCC [6, 8, 10-14].

In addition to dHBV particles, circulating HBV RNA-containing particles were reported in chronically infected patients. Recently, circulating HBV RNA has emerged as a promising biomarker for the therapeutic management of HBV infection [15]. The level of HBV RNA at baseline may predict the response to either interferon or nucleos(t)ide analogs (NUC) therapies, or inform on the risk of virological relapse at the end of treatment in both HBeAg-positive and -negative patients [16-30]. Furthermore, in untreated patients, the level of circulating HBV RNA has been correlated with both the transcriptional activity of cccDNA and HBV DNA viral load [23, 26, 27, 31-35]. Nucleocapsid containing HBV RNA was preferentially naked in the supernatant of HBV expressing cells and enveloped in the blood of infected patients [3].

The nature of HBV RNA in blood of infected patients remains a matter of debate. It has been reported both *in vitro* and *in vivo* that RNA-containing particles include mainly pgRNA (pregenomic or precore) or sp1RNA [15, 32]. Circulating HBV RNA may also be heterogeneous in size as a result of truncation, mainly at the 3' end [24]. In line with these data, high serum level of circulating pgRNA may result from a reverse transcription failure [24, 36]. Furthermore, subgenomic viral RNAs, and particularly HBx transcripts, have also been reported within circulating HBV particles [32].

It was recently suggested that HBV RNA levels might reflect the stage of liver disease [21, 26, 33, 37]. However, the mechanism involved in the synthesis of circulating RNA particles and their impact on the viral lifecycle remain unknown, especially considering their inability to generate a productive infection [38].

Overall, there are four main HBV nucleic acid forms (HBV_{naf}) that have been shown to circulate in the blood of chronically infected patients. Our study aimed to better define the diversity of these circulating forms in chronic HBV carriers and address its role during viral infection.

Patients and Methods

Patients

HBV infected patients (n=162, all positive for HBsAg), included between 2012 and 2016 in the French ANRS prospective and multi-centric cohort CO22 “HEPATHER” (NCT01953458), were selected to cover the different phases of the natural course of chronic HBV infection from HBeAg positive or negative hepatitis to cirrhosis and HCC. All patients were >18 years old, mono-infected patients and HIV negative. Fibrosis score was determined after hepatic biopsy or by non-invasive methods such as fibroTest® and fibroScan. The upper normal limit (N) for ALT was considered to be 50 UI/L. Clinical data are detailed in Table 1. Written informed consent was obtained from each patient before enrolment. Hepather protocol was conducted in accordance with the Declaration of Helsinki and French law for biomedical research and was approved by the “Comité de Protection des Personnes Ile de France 3” Ethics Committee (Paris, France) and the French Regulatory Authority.

HBV_{naf} quantification

To quantify both RNA and DNA in the same nucleic acid extract of plasma sample (140µl), we first used QIAamp Viral RNA® (Qiagen) to extract total nucleic acid forms. Eluates of purified nucleic acids were split in two for: i) direct quantification of DNA or ii) RNA quantification after digestion of DNA with RNase free DNase and further cleaned up using NucleoSpin® RNA isolation kit (Macherey-Nagel) prior to cDNA synthesis and qRT-PCR. The same process was applied to sample of cell culture supernatants.

Due to the lack of an international standard for HBV RNA quantification, the comparison of HBV RNA and DNA levels and thus the exact RNA/DNA ratio is difficult to assess [39]. However, the degree of uncertainty in the measurement of the ratio by qPCR applies the same degree in all tested samples.

Intracellular viral genomes were extracted with QIAamp DNA Blood kit (Qiagen) or NucleoSpin RNA isolation kit (Macherey-Nagel) according to manufacturer's instructions. Reverse transcription of RNA samples (0.2µg) was performed using SuperScript™ II Reverse Transcriptase and random hexamers (ThermoFisher Scientific). A control reaction in the absence of RT was achieved to ensure DNase efficiency. Quantitative PCR (qPCR) reactions were performed using 7500 Real-Time PCR System with Power SYBR™ Green Master Mix (Invitrogen) in the following conditions: 95°C for 10 min followed by 40 cycles at 95°C for 15 s, and 60°C for 60 s. HBV DNA (flDNA + defDNA) and RNA (pgRNA + sp1RNA) forms were quantified using pangenomic primers located at the core gene (forward 2265∧2285; reverse 2378∧2400, genotype D numbering). These qPCRs amplified both spliced and unspliced derived forms. In addition, sp1RNA and related defDNA were quantified using primers encompassing spliced sp1 sites (forward 2420∧2436; reverse 589∧611 genotype D numbering). Quantification of pgRNA and flDNA were deduced from the difference of both qPCR (Fig.S1).

HBV cccDNA quantification was conducted by qPCR according to the method of Rivière et al [40] using 7500 real-time PCR System as well as by digital droplet PCR (ddPCR) with the QX200 Droplet Digital PCR System (Bio-Rad). For ddPCR assays, the plates were loaded with DNA samples and required consumables into the Automated Droplet Generator to partition the samples around 20,000 droplets in 20 µl. PCR amplification was performed in a C1000 Touch thermal cycler (Bio-Rad, Hercules,

CA, USA) with the following amplification program: 5 min at 95 °C, 40 cycles of denaturation for 30 s at 95 °C and annealing for 60 s at 60 °C (ramping rate set to 2 °C/s), final incubation step for 10 min at 98 °C. Fluorescence amplitude threshold to distinguish positive from negative droplets was based on amplification of negative controls (water and uninfected cells). Primers used for qPCR and ddPCR reactions are indicated in Figure S1.

For *in vitro* infection (detailed in the supplementary material section), amounts of intracellular HBV genomes were normalized to the cell number using standard curve of albumin DNA quantification.

HBsAg and HBeAg levels were measured with HBeAgQuant and HBsAgQuant kits on Alinity-I analyzer (Abbott Diagnostics).

Data obtained from patients were studied by a principal component analysis and classified using a hierarchical clustering. Detailed procedures are indicated in the supplementary material section.

Statistical analysis

Continuous variables were compared using Mann-Whitney U test or Student’s T-test as appropriate. Pearson's chi-squared test was used to compare categorical variables, and correlations were assessed using Spearman test. Statistical analyses were performed on PRISM v7 software (GraphPad) and significant differences were indicated (p value < 0.05).

Results

Diversity of viral circulating genomes in treatment-naive patients with chronic hepatitis B

The 162 untreated HBV mono-infected patients were recruited from the French national HEPATHER cohort, according to the 4 clinical phases of HBV chronic infection (HBeAg positive or negative chronic infection (CIe+ or CIe-) and HBeAg positive or negative chronic hepatitis (CHe+ or CHe-)), as defined in EASL guidelines.[1] The patients' characteristics are shown in Table 1. Using an in-house pangenotypic qPCR, we found that HBV DNA was undetectable in blood of 46/162 (28%) patients, all included in the clinical CIe- group (Fig.S2). Using the same PCR method, we found that circulating viral RNA was present in 61/162 (38%) of them (7.3 ± 6.9 Log_{10} RNA copies/ml) (Fig.1A). All RNA-positive samples were also positive for DNA, and DNA level was higher in RNA+ as compared to RNA- patients (7.1 ± 6.4 Log_{10} *vs.* 4.0 ± 3.6 Log_{10} DNA copies/ml, respectively, $p<0.0001$). As previously reported, HBV RNA levels significantly correlated with HBV DNA ($r = 0.73$; $p < 0.0001$) and HBsAg levels (Fig.1A and S3A). Thus, we characterized the four main circulating HBV nucleic acid forms (HBV_{naf}) in blood samples: i) HBV spliced1 RNA (sp1RNA), ii) pregenomic RNA (pgRNA), iii) sp1-related defective DNA (defDNA) and iv) full-length DNA (flDNA). All were quantified by differential qPCRs. Circulating defDNA, pgRNA, and sp1RNA levels were all correlated with flDNA (Fig.1B-D). Furthermore, pgRNA and sp1RNA were positively correlated with each other ($r = 0.85$; $p < 0.0001$) (Fig.S3B) and with their corresponding DNA (fl or def DNA) forms (Fig.S3C).

In summary, the four main circulating HBV_{naf} could be detected in untreated patients, and circulating DNA was always detected in the RNA positive samples. Despite their correlation, probably in line with their common origin (pgRNA in infected cells), the range of their proportions has never been investigated.

Relationship between the proportion of each circulating HBV_{naf} and clinical features

We evaluated the ratio of the different HBV_{naf} in the patients who were positive for HBV DNA, *i.e.*, 116 out of 162. Samples with less than 3 Log_{10} of total circulating HBV_{naf} (n=18) were excluded, to ensure optimal sensitivity in the detection of each HBV_{naf} (Fig.1). The patients who were excluded mainly belonged to the CIe- group (Fig.S4). Overall, 98 of 116 HBV-replicative patients were studied. We performed a principal component analysis (PCA), in which a dot represents a patient, according to the proportion of each circulating HBV_{naf} (Fig.2A). Hierarchical clustering of this PCA identified 2 clusters (Fig.2B). Cluster 1 (C1, n=59) was defined by a predominance (>50%) of flDNA in all samples, whereas cluster 2 (C2, n=39) included various proportions of each viral form. In C2 patients, the major circulating form was pgRNA, flDNA or defDNA (77%, 18% or 5% of samples, respectively) (Fig.2B). Noteworthy, sp1RNA, which was detected in 43% of samples, was never the main form in our cohort (Fig.2B).

The classification of patients according to the chronic phases of HBV infection [1], showed that both clusters were represented at each stage (Fig.2C, Table S1). However, the distribution of C1 and C2 patients, was significantly different across the four different phases ($p<0.05$). Indeed, CIe+, CIe- or CHe- patients were preferentially included in C1 (in 70%, 77% and 72%, respectively). By contrast, 60% of CHe+

patients were included in C2. In keeping with this finding, C2 patients were more frequently associated with an HBeAg (+) status ($p<0.05$). The association between HBeAg detection and circulating HBV_{naf} diversity was confirmed, independently of the hierarchical clustering classification (Fig.S5).

Therefore, HBV_{naf} diversity is observed at all phases of chronic HBV infection but it is predominant in HBeAg(+) patients with chronic hepatitis.

Analysis of circulating HBV_{naf} diversity according to viral markers

In line with the repartition of C1 *vs.* C2 patients according to the clinical status (Table S1), HBsAg and HBV flDNA levels were significantly lower in C1 as compared to C2 patients ($1.3x10^4 \pm 2.6\ x10^3$ *vs.* $3.0x10^4 \pm 7.6\ x10^3$ IU/ml, $p<0.01$) and (6.6 ± 6.2 *vs* 7.0 ± 6.4 Log_{10} copies/ml, $p<0.01$), respectively (Fig.3A). Overall, these data reinforce the relationship between the level of HBV expression (flDNA) and the occurrence of HBV_{naf} diversity.

In order to decipher this relationship, we compared the proportion of HBV_{naf} in patients with low ($<10^5$ copies/ml) or high ($\geq 10^5$ copies/ml) HBV replication. Full-length DNA levels showed a similar distribution and mean value across C1 and C2 in low (4.1 ± 3.7 *vs* 4.1 ± 3.5 Log_{10} copies/ml) and high (7.1 ± 6.6 *vs* 7.2 ± 6.5 Log_{10} copies/ml) replication groups (Fig.3B). We determined the percentage of circulating pgRNA over total HBV_{naf} level in the C1 population of both replicative groups. This proportion was significantly greater in the high than in the low replication groups (22.1% ± 2.5% *vs.* 4.1% ± 1.8%, $p<0.0001$). By contrast, this percentage was equally high in C2 patients, irrespective of the replication level (67.5% ± 9.5% *vs.* 57% ± 2.9% in low and high replicative groups, respectively) (Fig.3C). Consistent with these data, we found no correlation between circulating pgRNA and flDNA levels in C2 patients with low

replicative rate, unlike the 3 other groups (Fig.S6). Similar results were obtained when the percentage of circulating sp1RNA+defDNA was taken into account instead of pgRNA (Fig.3C, right panel).

Therefore, whereas HBV replication levels positively correlate with the diversity of circulating HBV_{naf} in C1 patients, our data suggest that an additional mechanism(s) drive(s) viral diversity in C2 patients, whatever the level of viral replication.

Impact of HBV strain on the diversity of circulating HBV_{naf}

We sought to determine if the viral diversity might depend on the infectious viral strain from each patient. We performed *in vitro* phenotypic assays to address this relationship. Seven plasma samples (samples 1 to 6 from C2 and sample 7 from C1 patients) were used to infect dHepG2-NTCP cells at 40 m.o.i. ($3.2x10^6$ copies of $RNA_{(pg+sp1)}$ plus $DNA_{(fl+def)}$ / 80000 cells per well) (Fig.4A). Plasma 1 and 2 contained 9% and 12% of flDNA respectively, while this proportion was higher and up to 60% in the other samples (Fig.4A). At day 10 post-inoculation, the levels of intracellular cccDNA were lower in cells exposed to plasma 1 and 2, as expected, if one considers that only flDNA should be able to generate a productive infection (Fig.4B). This result was confirmed by ddPCR (Fig.S7). However, infection with samples 1 and 2 led to higher proportion and copy numbers of intracellular HBV flDNA and defDNA as compared to cells infected with the other samples (Fig.4C). Furthermore, this high amount of intracellular HBV DNA was associated with a low level of pgRNA (Fig.4C). In cells infected with samples 3-7, pgRNA was the main intracellular HBV_{naf} form and its level positively correlated with that of cccDNA. In all cases, secreted viral particles at 10 days post-inoculation contained mainly flDNA (>80%), suggesting that circulating HBV_{naf} diversity is not a characteristic of a specific HBV strain (Fig.4D). Of note, these

amounts in the supernatant were consistent with the intracellular viral DNA content, except for samples 1 and 3 (Fig.4E). HBV genome sequence analysis showed that infectious genotype and mutation profile did not explain the differences observed between patients (Table 2 and S2).

Altogether, these results do not support a significant role of viral strain in the circulating HBV_{naf} diversity, but they suggest an impact of diversity on viral replication efficiency, independently of the level of cccDNA.

pgRNA-containing HBV particles modulate viral replication *in vitro*

We assessed the role of pgRNA-containing HBV particles in viral replication. To do so, we collected the supernatant of a cell line stably-expressing HBV virions, which were untreated (HBV_{sn}) or treated with Tenofovir ($HBV_{sntreated}$) for 10 days. As illustrated in Figure 5A, Tenofovir decreased the level of flDNA without altering the pgRNA in the culture supernatant. Then, supernatants of non-treated and treated cells were mixed to enrich HBV_{sn} with pgRNA-containing particles at a ratio of 1 to 5, respectively, before HepG2-NTCP infection (Fig.5B). At day 10 post-inoculation, residual DNA particles, produced in $HBV_{sntreated}$, were unable to efficiently generate cccDNA, as opposed to HBV_{sn} (Fig.5C). The enrichment of an inoculum with pgRNA (mix inoculum) did not modify the cccDNA level in infected cells (Fig.5C). As previously suggested in our infection assay using sample patients, pgRNA particles enrichment significantly increased the amount of viral DNA in intra- and extra-cellular compartments, while reducing intracellular pgRNA, in HBV_{mix} compared to HBV_{sn} infected cells (Fig. 5D and 5E).

These data indicated that pgRNA-containing HBV particles may interfere with infection at a post-cccDNA formation level.

Discussion

HBV is a hepatotropic virus and a member of the *Hepadnaviridae* family, whose infectious particle contains a partially double-stranded circular DNA genome of 3.2 kb (flDNA). It has long been thought that flDNA was the only HBV_{naf}, present in virion, in the blood of chronically infected patients [2]. However, numerous data have illustrated the presence of diverse circulating HBV_{naf}, either DNA or RNA, of genomic or sub-genomic (full-length or truncated). There are numerous studies that quantified circulating pgRNA [16-19, 22, 24, 25, 30, 34] or defective DNA generated from spliced1 RNA [7, 8, 10-14], but the overall diversity of circulating HBV_{naf} had remains poorly explored. Strategies, which can be used to investigate this viral diversity include cloning/sequencing, 5'RACE-PCR or next-generation sequencing assays [41].

In this study, we applied a pangenotypic qPCR approach to quantify circulating HBV_{naf}, *i.e.*, flDNA, defDNA, pgRNA and sp1RNA, in the same nucleic extract of plasma collected from patients, at different phases of chronic liver disease. Full-length DNA and related pgRNA were the most frequent forms, whereas spRNA and related defDNA were commonly detected but rarely predominant. Strong correlations between these forms were observed, as previously reported for some of them [8, 10, 13, 23].

Our classification of patients according to the proportion of each circulating form defined 2 clusters: C1 in which the presence of flDNA is almost exclusive, and C2 in which HBV_{naf} diversity dominates. Cluster 1 was preferentially associated with CIe+, CIe- or CHe-, whereas cluster 2 was more frequently observed in CHe+ clinical phase. Based on the clinical distribution of clusters and their relationship to HBeAg and flDNA, the level of HBV replication appears as a key driver of HBV_{naf} diversity. This was observed in patients with a C1 profile in which the proportion of circulating pgRNA

positively correlated with replication markers. However, the existence of an additional driver of HBV_{naf} diversity, unrelated to the level of replication, was supported by the significant higher proportion of circulating pgRNA and sp1RNA+defDNA forms in C2 compared to C1 in the low and high replicative groups. In the same line, the low prevalence of C2 in CIe+ patients (high HBV replication) suggests that this regulatory factor of HBV_{naf} diversity may be associated with either the liver disease or the progression of HBV chronic infection. Liver disease parameters (ALT, fibrosis score), showed no significant differences between C1 and C2, in our study (Table S1). Furthermore, impact of viral strains on HBV_{naf} diversity was not supported by our genotyping and *in vitro* infection assays.

Interestingly, data obtained *in vitro* suggest an influence of circulating HBV_{naf} diversity on the dynamic of the viral replication cycle of infected cells, independently of the viral genotype or viral mutations. Viral particles containing HBV_{naf} other than HBV 3.2 kb flDNA are hampered in their ability to generate a productive infection. Indeed, deletion of viral genome, through alternative splicing, in spliced RNA or spliced-derived DNA particles cause a constitutive failure to achieve viral cycle [7, 9]. Regarding pgRNA particles, their inability to establish infection was demonstrated *in vitro* [38]. Unlike defDNA particles that may lead to the production of additional viral proteins such as HBSP, circulating pgRNA particles have no known function in the HBV replication cycle, but they may serve as a biomarker for cccDNA activity and antiviral response [3, 7, 26, 33, 42]. It was previously reported that HBx transcripts might be present in circulating RNA particles [32]. Thus, HBx transcripts could contribute to enhanced viral replication, through Smc5/6 degradation. However, it was also reported that HBx sequence located at the 3' end of pgRNA was frequently deleted in RNA-containing particles [24, 38]. Note that in inoculum used for *in vitro* infection assay, quantification using HBx

primers showed a reduced amount compared to quantification of pgRNA (data not shown). Although we could not exclude the presence of HBx RNA in particles, a high proportion of pgRNA in an inoculum may, on its own, affect viral replication in infected cells. To investigate this hypothesis, we spiked an HBV inoculum with pgRNA-containing particles, which were obtained from the supernatant of HBV-producing cells upon treatment with NUC. Because of NUC incorporation into DNA, residual circulating viral DNA was not efficient to generate a productive infection, as previously reported [43]. Cells infected with inoculum enriched in pgRNA demonstrated no increase of cccDNA level at day 10 post-inoculation but, surprisingly, led to a significant increase of intracellular and extracellular HBV DNA. Regarding the similar viral density and the structure of viral envelope between HBV pgRNA virion-like and Dane particles [26, 36], we could easily assume that these particles may enter hepatocytes. However, their failure to ensure a productive infection suggests an impairment in cccDNA synthesis [38]. One hypothesis is that entry of RNA-containing virions may deliver viral components (transcripts and/or proteins) that could act in trans on the replication of flDNA. These imported factors, such as viral polymerase, HSP90 or RBM24 [44], are involved in pgRNA packaging or reverse transcription and could therefore guide nascent pgRNA towards viral DNA synthesis. Furthermore, considering the frequent 3' truncation of circulating HBV pgRNA, this viral RNA may be engaged in the replication process inside nucleocapsid [24, 38]. Such abortive replication complex would be unable to be translated after hepatocyte infection and therefore to generate alone a productive infection, as previously reported [38]. However, trans-complementation by flDNA virus may contribute to the recovery of replication for these complexes, leading to an enhancement of viral DNA synthesis, independently of cccDNA. Thus, circulating viral

pgRNA, may constitute an additional mechanism which interferes with viral replication in chronic infected patients.

Taken together, our data provide evidence for an evolution of circulating HBV_{naf} diversity, during chronic infection, independently of the viral strain. The viral diversity may be a useful biomarker for the management of chronically infected patients, as previously reported [16-30]. It may also participate to the HBV persistence through enrichment of the viral proteome by spliced forms [9] or, as shown here, an unconventional regulation of the viral replication by pgRNA particles. Further clinical investigations, including a follow-up of infected patients, is needed to better appreciate the regulation and the consequence of circulating HBV_{naf} diversity in the course of chronic infection.

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Statements & Declarations

Data availability: The work described has not been published before, it is not under consideration for publication anywhere else and this submission was approved by all co-authors.

Animal research: This article does not contain any studies with animals.

Consent to Participate: Written informed consent was obtained from each patient before enrolment. Hepather protocol was conducted in accordance with the Declaration of Helsinki and French law for biomedical research and was approved by the “Comité de Protection des Personnes Ile de France 3” Ethics Committee (Paris, France) and the French Regulatory Authority.

Consent to publish: All authors agreed with the content and gave explicit consent to submit this work.

Clinical trial registration: Patients were included from a prospective multicenter French national cohort (ANRS CO22 Hepather, NCT01953458).

Author contributions:

Conceptualization: SP.

Experiments and procedures: SJ, SA, BP, LB, G-DM, LL, SN, NC, SP.

Data analysis: SJ, SA, KD, SP.

Writing, reviewing and editing: SJ, SA, BP, LB, AJ, G-DM, LL, DC, CF, PS, FH, SN, NC, HC, KD, SP.

Conflict of interest: Jules Sotty, Pierre Bablon, Bouchra Lekbaby, Jeremy Augustin, Morgane Girier-Dufournier, Lucas Langlois, Celine Dorival, Fabrice Carrat, Stanislas Pol, Helene Fontaine, Nazim Sarica, Christine Neuveut, Chantal Housset, Dina Kremsdorf, Aurelie Schnuriger and Patrick Soussan declare that they have no current financial arrangement or affiliation with any organization that may have a direct influence on the present work.

The ANRS CO22 HEPATHER cohort is sponsored and funded by INSERM- ANRS and conducted in collaboration with Association Française pour l'étude du Foie (AFEF). The cohort received supports from Agence Nationale de la Recherche (ANR), Direction Générale de la Santé (DGS), MSD, Janssen, Gilead, Abbvie, BMS, and Roche. The public/private partnership is built in total transparency through a specific contract. The pharmaceutical companies are not involved in scientific decisions. The sponsor did not contribute to the study design and drafting of the study. The sponsor played no role in data collection, data analysis or data interpretation. The other funding sources played no role in study design, data collection, data analysis, data interpretation, or drafting the study.

CF had full access to all data in the cohort study.

Funding : This work was supported by funding from Agence Nationale de la Recherche sur le Sida et les Hépatites (ANRS) (grant numbers ECTZ 103985 and ECTZ 163186) and from Fondation pour la Recherche Médicale (FRM) (grant number EQU202003010517).

Figure Legends

Figure 1: HBV_{naf} circulating diversity in untreated patients

Correlations observed between (A) HBV DNA / RNA, (B) defDNA / flDNA, (C) pgRNA / flDNA and (D) sp1RNA / flDNA. Viral amount of each viral circulating genome form (mean ± SE): flDNA: 6.6 ± 5.9 Log_{10} copies/ml; defDNA: 5.6 ± 5.2 Log_{10} copies/ml; pgRNA: 6.9 ± 6.4 Log_{10} copies/ml; sp1RNA: 5.9 ± 5.6 Log_{10} copies/ml. HBV_{naf}: HBV nucleic-acid forms, flDNA: full-length DNA, defDNA: defective DNA, pgRNA: pregenomic RNA, sp1RNA: spliced 1 RNA. Spearman correlation assay.

Figure 2: Hierarchical clustering of patients according to HBV_{naf} circulating diversity and distribution across chronic infection phases

(A) Principal component analysis (PCA) showing the repartition of patients according to each variable (proportion of each viral form that constitutes the HBV_{naf}). The correlation circle in the center of the figure explains the spatial distribution of patients on the PCA. Positive (or negative) correlated variables are plotted in the same (or the opposite) quadrant. Dim1: dimension 1 and Dim 2: dimension 2. The percentage in brackets indicates the explained variance between patients in each axis of the PCA. (B) Hierarchical clustering identified 2 distinct clusters of patients (clusters 1 and 2) characterized by different proportions of HBV genomes. (C) Distribution of patients at the different phases of chronic HBV infection according to each cluster. Cluster 1 was indicated in red and Cluster 2 in blue. The repartition of C1 / C2 (n/n) patients was (6/3) in CIe+, (15/22) in CHe+, (10/3) in CIe- and (28/11) in CHe-, respectively. Significant difference of the clinical repartition for each cluster of patients was determined using

Chi Square test ($p<0.05$). HBV chronic infection phases: CIe+: HBeAg positive Chronic Infection, CHe+: HBeAg positive Chronic Hepatitis, ICe-: HBeAg negative Chronic Infection, CHe-: HBeAg negative Chronic Hepatitis.

Figure 3: Analysis of viral features in both clusters of patients with HBV chronic infection

(A) Distribution of HBsAg quantification values (left panel) and flDNA viral load (right panel) in patients included in cluster 1 and 2. (B) Distribution of flDNA viral load in low ($<10^5$ copies/ml) and high ($>10^5$ copies/ml) replicative patients included in cluster 1 and 2. (C) Percentage of circulating pgRNA forms (left panel) or sp1RNA + defDNA (right panel) in low and high viral replicative patients for C1 and C2 (mean ± SE).

flDNA: full-length DNA, defDNA: defective DNA, pgRNA: pregenomic RNA, sp1RNA: spliced 1 RNA. Means are indicated on scatter plot. Mann-Whitney U (quantitative) or Pearson's Chi-Square test (qualitative) significance was considered when: *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 4: infection of dHepG2-NTCP with blood samples containing variable proportions of each circulating HBV_{naf}

(A) HBV genome diversity quantified in seven blood samples used for dHepG2-NTCP infection at m.o.i 40 ($3.2x10^6$ copies of DNA + RNA genomes/well) (A). Ten days post-infection, nucleic acids from intra- and extracellular compartments of infected cells were extracted to quantify HBV DNAs, RNAs and (B) cccDNA. (C) Proportion and (D) amount of intracellular HBV nucleic acids (DNA and RNA) at 10 days post-infection for each sample. (E) Proportion and (F) amount of HBV circulating genomes in

supernatant of infected cells, 10 days post-infection. HBV_{naf}: HBV nucleic-acid forms, flDNA: full-length DNA (light green), defDNA: defective DNA (dark green), pgRNA: pregenomic RNA (light blue), sp1RNA: spliced 1 RNA (dark blue). For each viral form data were presented as mean ± SD, n=3.

Figure 5: infection of dHepG2-NTCP using an inoculum enriched with pgRNA particles.

(A) HepG2 cells stably expressing HBV were treated with Tenofovir disoproxil fumarate (TDF, 10µM) for 10 days. As indicated, cells were treated 3 times after medium change (arrows). Cell supernatants were collected in non-treated cells (HBV_{sn}) or at 10 days post-treatment ($HBV_{sntreated}$). (B) Ultra-centrifuged HBV_{sn} and $HBV_{sntreated}$ supernatants were used to infect dHepG2-NTCP at 40 m.o.i ($3.2x10^6$ copies of all viral forms/ml for HBV_{sn}) (HBV DNA from $HBV_{sntreated}$ (green and white hatched bar) was not considered). Mix inoculum, a combination of 1 equivalent HBV_{sn} and 5 equivalents $HBV_{sntreated}$ and thus containing 90% of HBV pgRNA forms, was used to infect dHepG2-NTCP cells (HBV DNA from $HBV_{sntreated}$, was indicated as green and white hatched bar). (C) cccDNA copies in cells infected with $HBV_{sn,}$ $HBV_{sntreated}$ or mix inoculum. Proportion and amount of intra- (D) and extracellular (E) HBV nucleic acids in cells infected with $HBV_{sn,}$ $HBV_{sntreated}$ or mix inoculum. flDNA: full-length DNA (light green), defDNA: defective DNA (dark green), pgRNA: pregenomic RNA (light blue), sp1RNA: spliced 1 RNA (dark blue). For each viral form data were presented as mean ± SD, n=3. Student‘s t-test was used to assess differences compared to HBV_{sn} values, significance was considered when *p<0.05, **p<0.01, ***p<0.001.

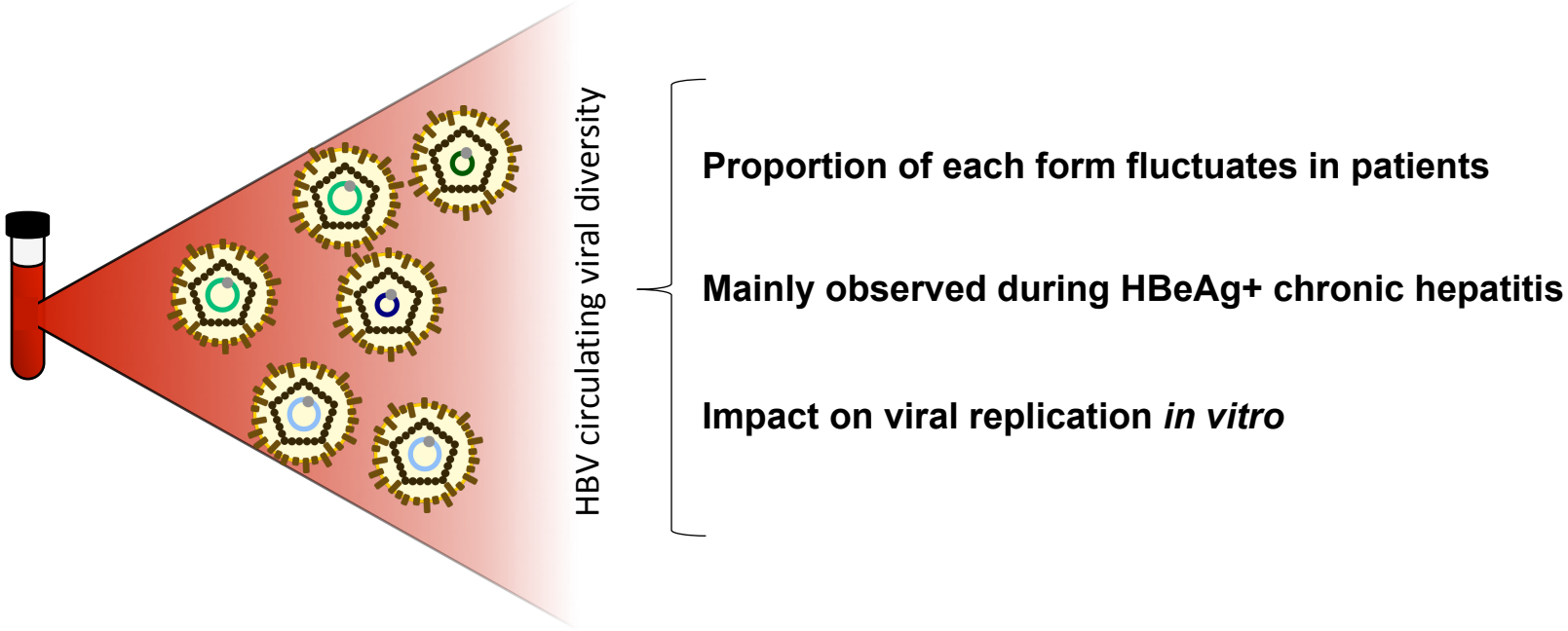
Table 1: clinical characteristics of patients included

Clinical and virological of patients with HBV chronic infection (n=162) from the HEPATHER cohort. HBV chronic infection phases were defined according to the EASL guidelines. Data are expressed as median and range.

Table 2: Genotyping of HBV samples used for infection assays.

HBV genotype and mutations, involved in the modulation of viral replication, were reported for the 7 patient samples used for infection assay. Polymerase mutations are indicated in amino-acids whereas they are reported in nucleic acids for the other genes. Genotype and mutations were obtained after sequence analysis. WT: wild-type, MUT: mutant, WT/MUT: double-population wild-type and mutant.

graphical abstract



Figure

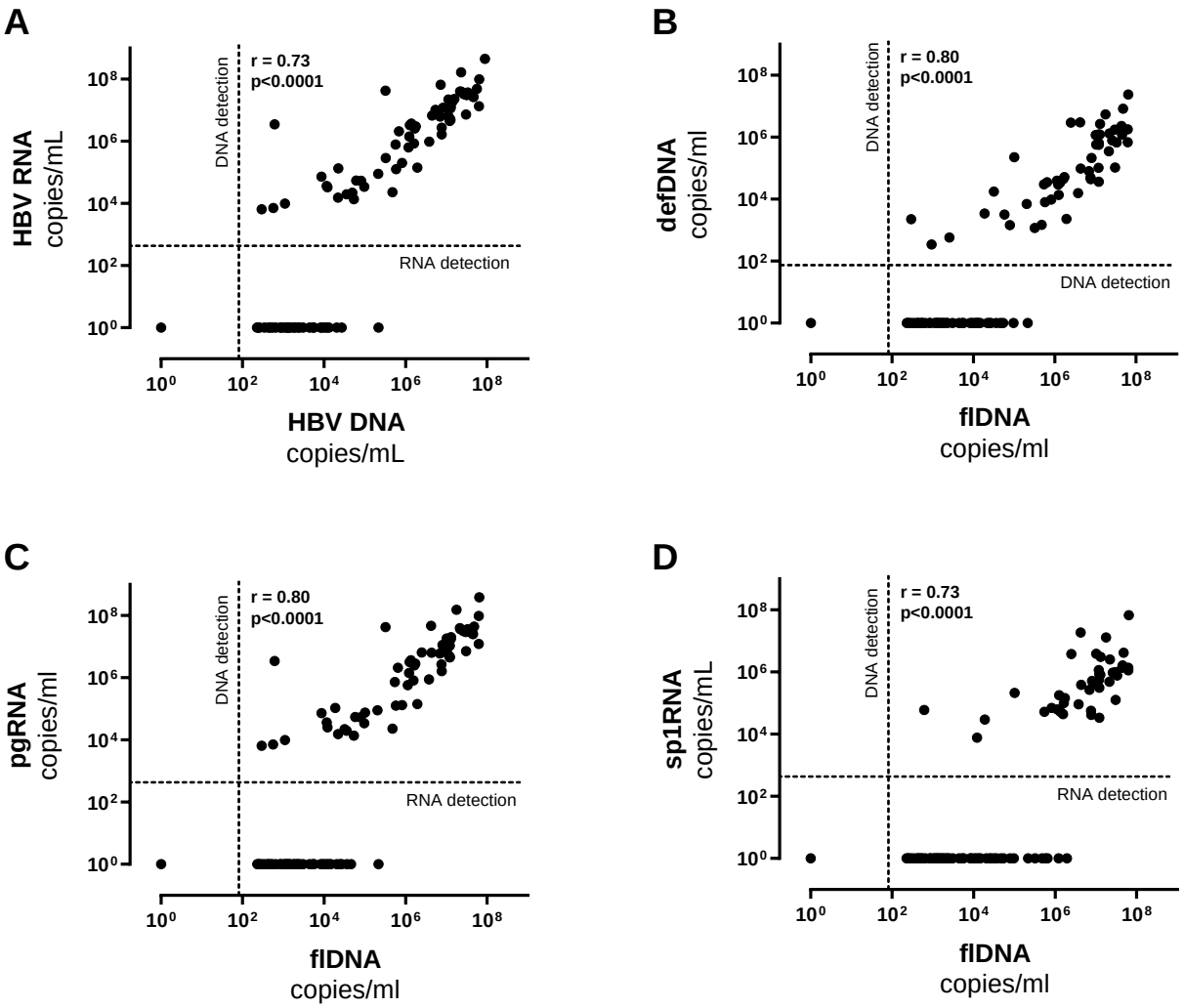
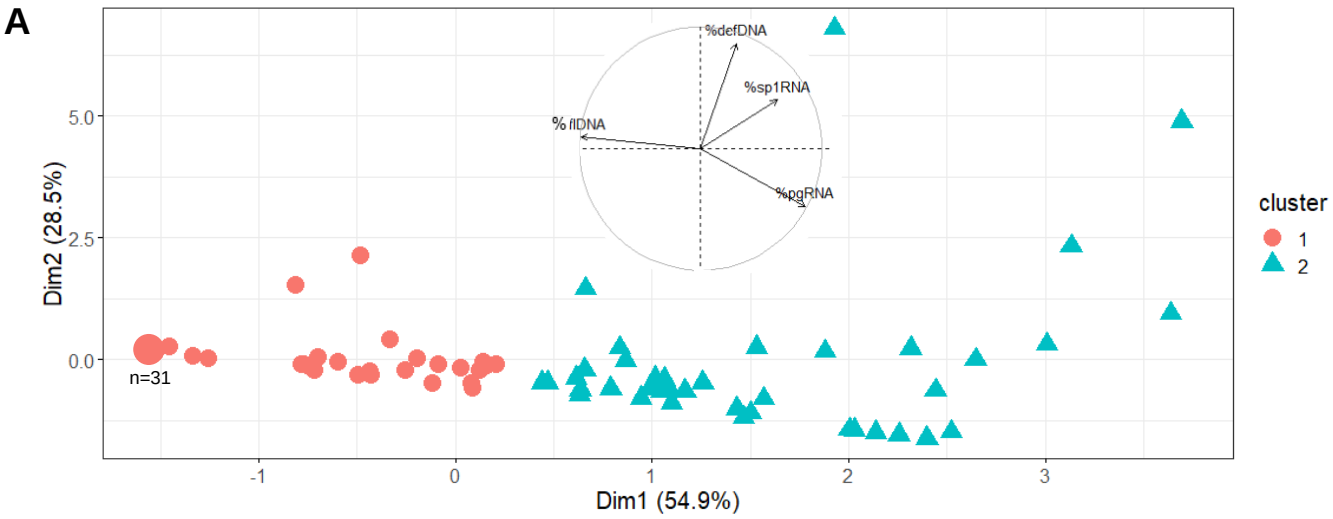
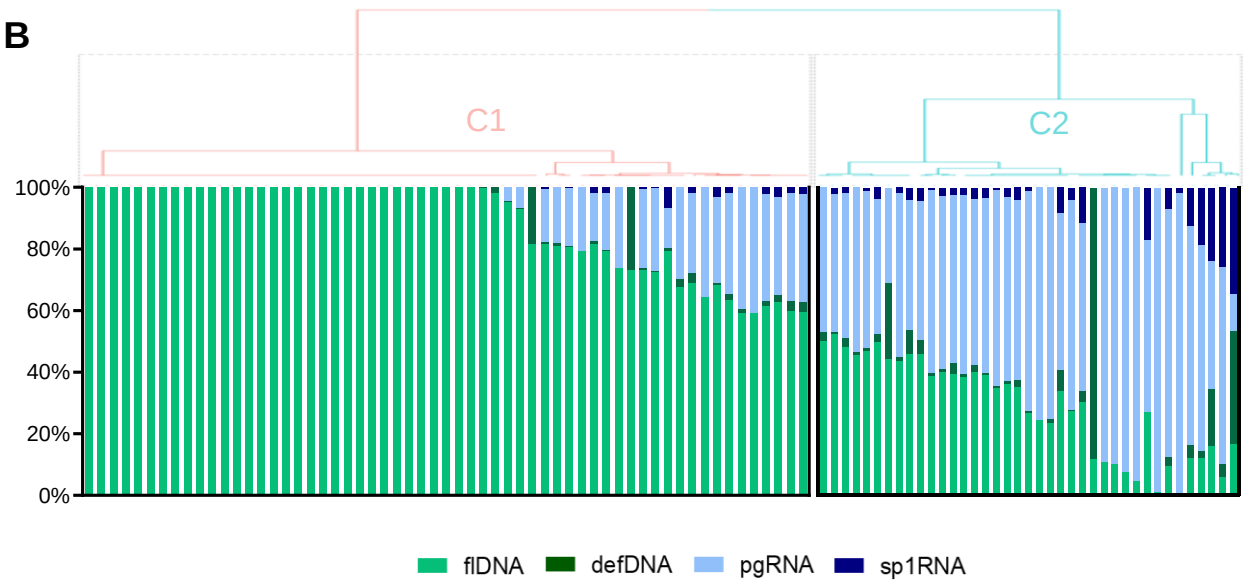


Figure 1


A
%defDNA
%sp1RNA
% flDNA
%pgRNA
5.0
2.5
0.0
Dim2 (28.5%)
n=31
-1
0
1
2
3
Dim1 (54.9%)
cluster
1
2


B
C1
C2
100%
80%
60%
40%
20%
0%
flDNA
defDNA
pgRNA
sp1RNA

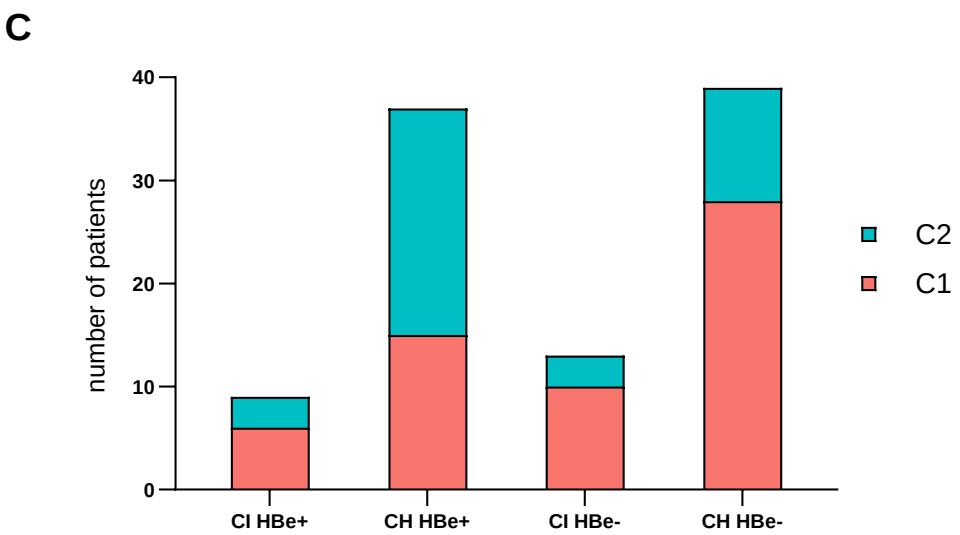

C
40
30
20
10
0
number of patients
Cl HBe+
CH HBe+
Cl HBe-
CH HBe-
C2
C1

Figure 2

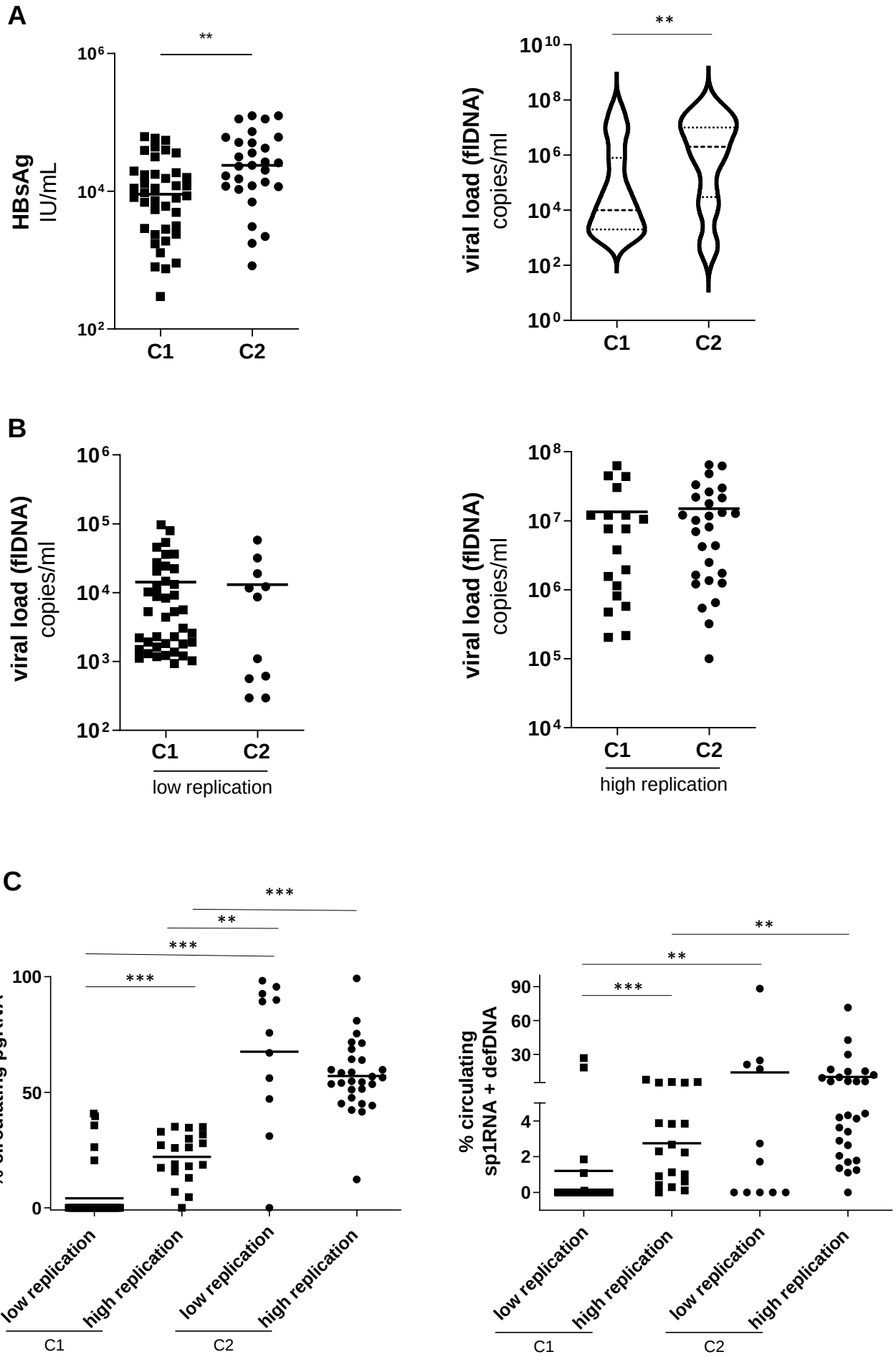

A
**
HBsAg
IU/mL
10⁶
10⁴
10²
C1
C2
**
viral load (flDNA)
copies/ml
10¹⁰
10⁸
10⁶
10⁴
10²
10⁰
C1
C2
B
viral load (flDNA)
copies/ml
10⁶
10⁵
10⁴
10³
10²
C1
C2
low replication
viral load (flDNA)
copies/ml
10⁸
10⁷
10⁶
10⁵
10⁴
C1
C2
high replication
C
***
**
***
***
% circulating pgRNA
100
50
0
low replication
high replication
low replication
high replication
C1
C2
**
**
***
% circulating
sp1RNA + defDNA
90
60
30
4
2
0
low replication
high replication
low replication
high replication
C1
C2

Figure 3

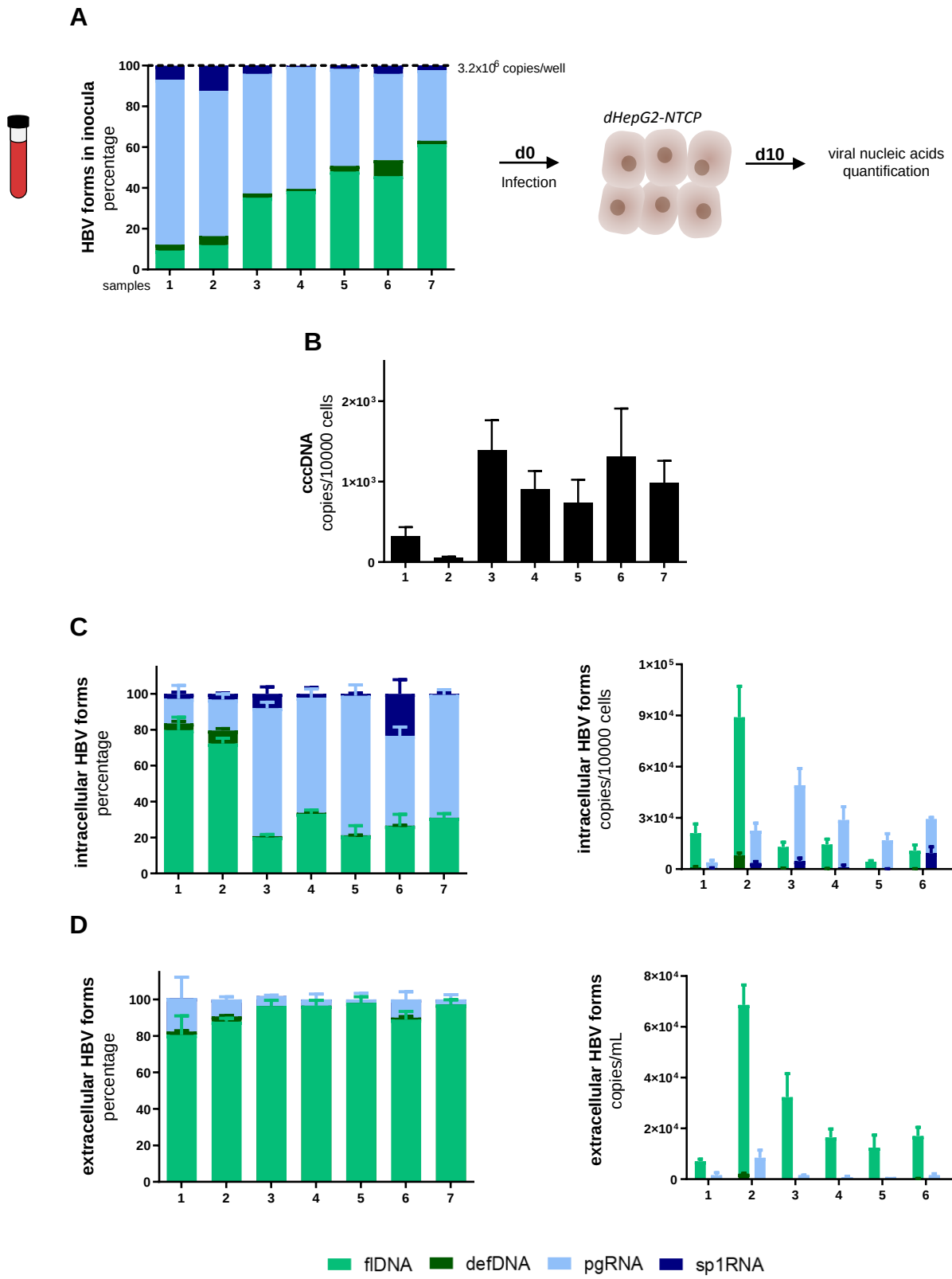

A
HBV forms in inocula
percentage
samples
3.2x10^6 copies/well
d0
Infection
dHepG2-NTCP
d10
viral nucleic acids
quantification
B
cccDNA
copies/10000 cells
C
intracellular HBV forms
percentage
intracellular HBV forms
copies/10000 cells
D
extracellular HBV forms
percentage
extracellular HBV forms
copies/mL
flDNA
defDNA
pgRNA
sp1RNA

Figure 4

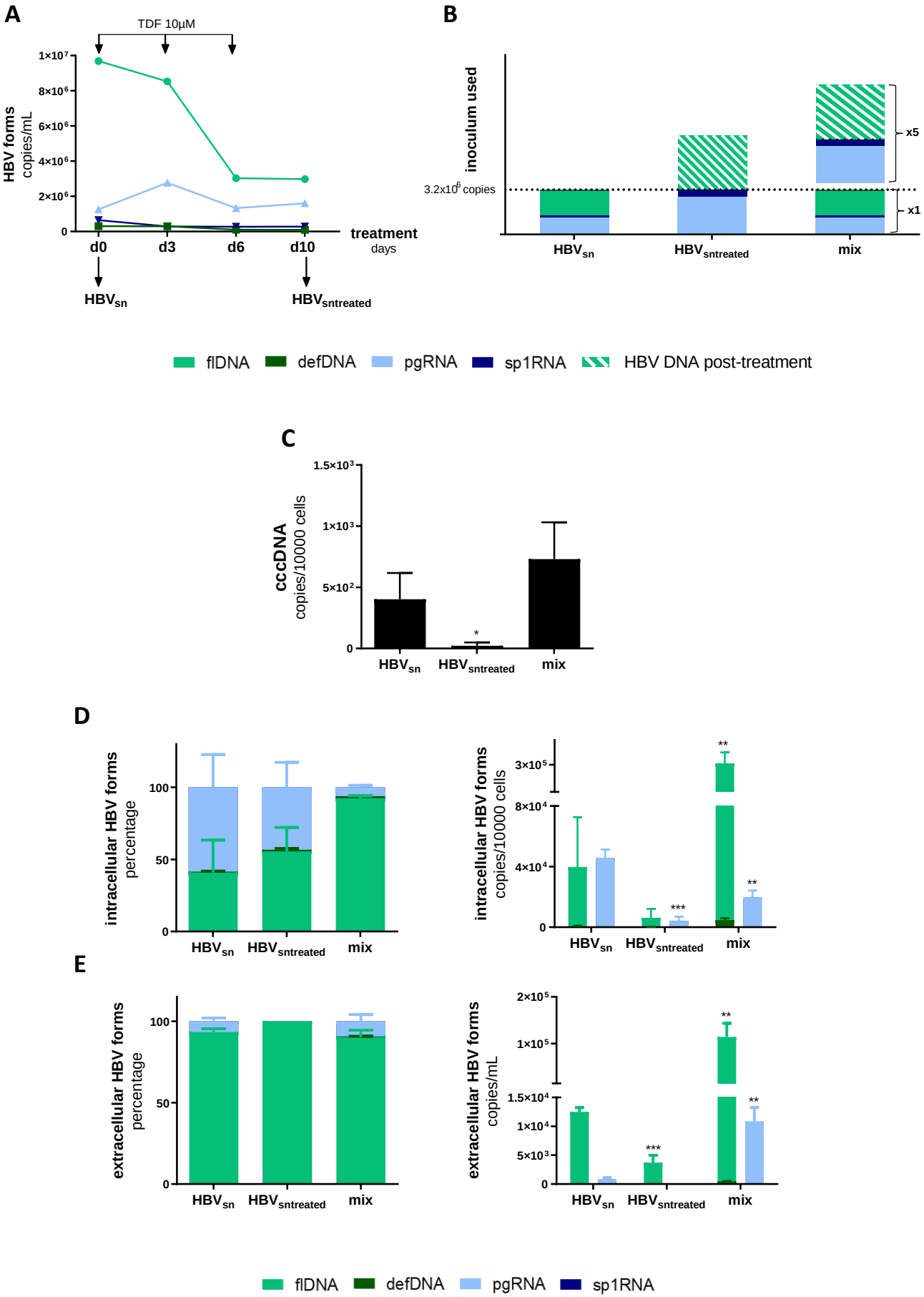

A
TDF 10µM
HBV forms
copies/mL
1×10⁷
8×10⁶
6×10⁶
4×10⁶
2×10⁶
0
d0
d3
d6
d10
treatment
days
HBVsn
HBVsntreated
B
inoculum used
3.2x10⁶ copies
x5
x1
HBVsn
HBVsntreated
mix
flDNA
defDNA
pgRNA
sp1RNA
HBV DNA post-treatment
C
cccDNA
copies/10000 cells
1.5×10³
1×10³
5×10²
0
*
HBVsn
HBVsntreated
mix
D
intracellular HBV forms
percentage
100
50
0
HBVsn
HBVsntreated
mix
intracellular HBV forms
copies/10000 cells
3×10⁵
8×10⁴
4×10⁴
0
**
***
**
HBVsn
HBVsntreated
mix
E
extracellular HBV forms
percentage
100
50
0
HBVsn
HBVsntreated
mix
extracellular HBV forms
copies/mL
2×10⁵
1×10⁵
1.5×10⁴
1×10⁴
5×10³
0
**
***
**
HBVsn
HBVsntreated
mix
flDNA
defDNA
pgRNA
sp1RNA

Figure 5

	Chronic Infection HBe+ (n=9)	**Chronic Hepatitis HBe+** (n=38)	**Chronic Infection HBe-** (n=72)	**Chronic Hepatitis HBe-** (n=43)	**Total** (n=162)
Age (years) *median (range)*	29 (20 - 37)	28 (18 - 78)	43 (19-77)	40 (22 - 83)	38 (18 - 83)
Sex ratio *(% male)*	34	64	54	64	59
Virology					
HBV DNA *median (range) copies/mL*	$2.7x10^7$ ($2.2x10^4$ - $6.4x10^7$)	$4.1x10^6$ ($5.2x10^2$ – $8.8x10^7$)	0.0 (0.0 – $1.5x10^4$)	$1.3x10^4$ ($2.3x10^2$ – $6.4x10^7$)	$1.9x10^3$ (0.0 – $8.8x10^7$)
HBsAg *median (range) IU/mL*	$4.0x10^4$ ($1.2x10^4$ – $1.1x10^5$)	$2.8x10^4$ ($1.8x10^3$ – $1.3x10^5$)	$4.8x10^3$ ($5.4x10^2$ – $2.6x10^4$)	$5.5x10^3$ (5.0 – $2.7x10^4$)	$1.2x10^4$ (5.0 – $1.3x10^5$)
Liver disease					
ALT *median (range) IU/mL*	25 (11 - 38)	73 (18 - 388)	28 (9 – 293)	67 (22 - 1861)	43 (9 - 1861)
Fibrosis score *(n=102)*					
F0 – F2	100%	70%	100%	59%	79%
F3 – F4	0%	30%	0%	41%	21%
HCC *post-inclusion*	0%	0%	0%	2.4%	0.6%

Table 1

Patient	Genotype	Polymerase	Core promoter	PreC/C		HBeAg	qHBeAg (PEIU/mL)	Envelope	qHBsAg (IU/mL)
		L180M, A181V, M204V, N236T, M250V	*A1762T/G1764A*	*G1896A*	*G1899A*			*ATG PreS1/PreS2/S*	
1	D	WT	WT	WT/MUT	WT	+	3	WT	3.6×10^4
2	A	WT	WT	WT	WT	+	176	WT	1.3×10^5
3	D	WT	WT	WT	WT	+	1347	WT	1.1×10^5
4	D	WT	WT/MUT	WT/MUT	MUT	-	0	WT	1.1×10^4
5	C	WT	WT	WT	WT	+	1453	WT	5.0×10^4
6	D	WT	WT	WT	WT	+	154	WT	1.3×10^5
7	C	WT	WT	WT	WT	+	1481	WT	4.4×10^4

Table 2