Intrahepatic quantification of HBV antigens in chronic hepatitis B reveals heterogeneity and treatment-mediated reductions in HBV core-positive cells

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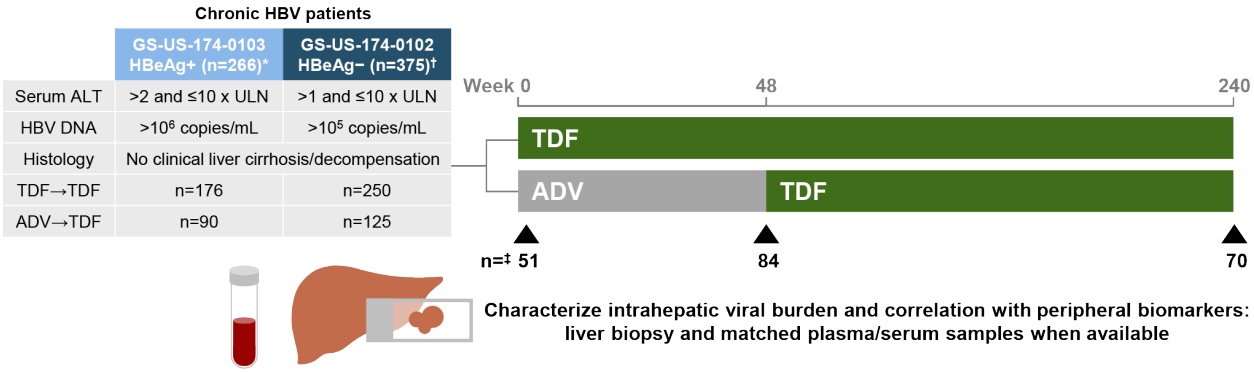

Fig. S1. Schematic of study design.

Longitudinal liver biopsies were obtained from subjects enrolled in GS-US-174-0102 (HBeAg-) and GS-US-174-0103 (HBeAg+). Subjects from both studies were randomized 2:1 to receive TDF or ADV for 48 weeks. Optional core needle liver biopsies were collected at baseline, and Week 44-48 (referred to as “Wk48”). Subjects who completed 48 weeks of treatment and provided a liver biopsy at Wk48 were given the option to begin open-label treatment with TDF for up to 7 additional years. A third optional liver biopsy was collected at Week 240 in subjects who continued treatment. Liver biopsies were stained by multiplex IF and, when available, paired plasma/serum samples were analyzed for peripheral biomarkers. *ClinicalTrials.gov NCT00116805; †NCT00117676; ‡No. of liver biopsies analyzed. ULN, upper limit of normal.

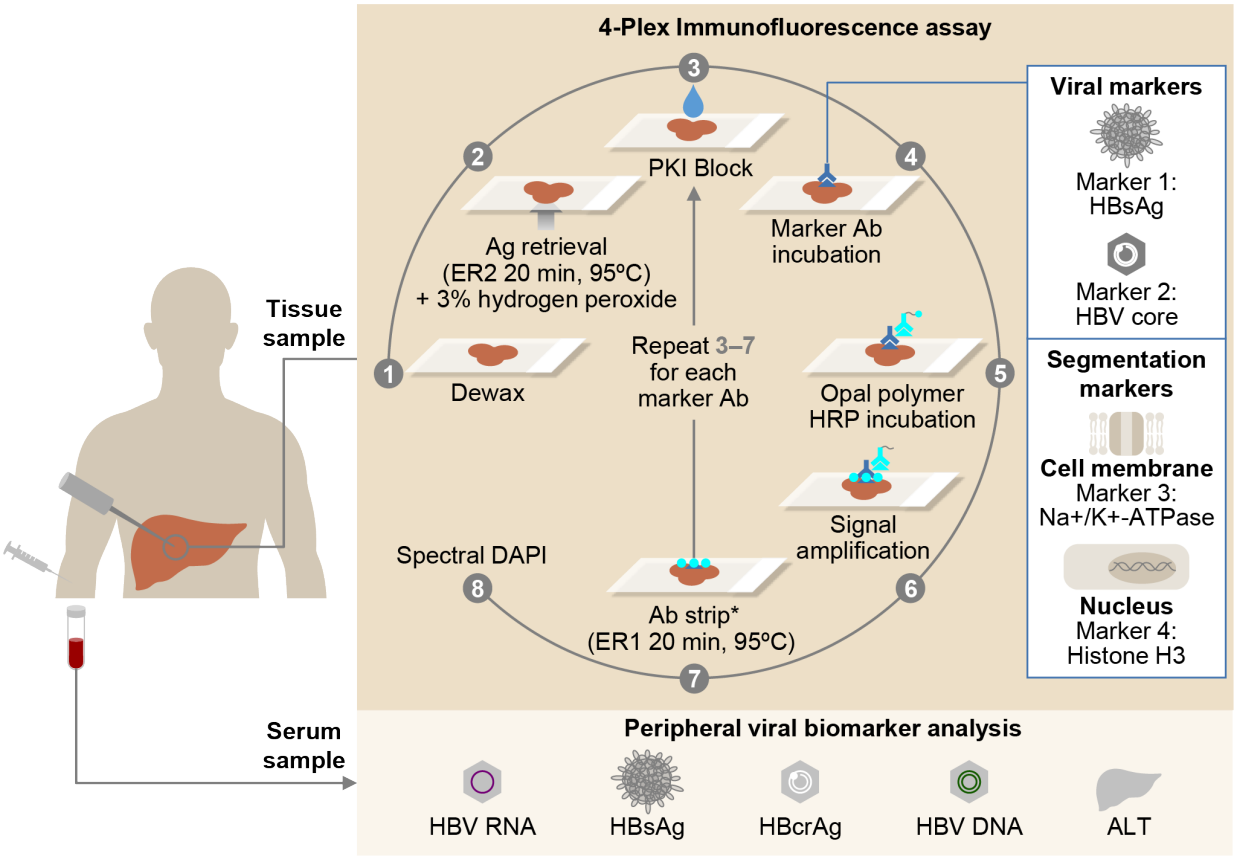

Fig. S2. Overview of assays performed on serum and tissue samples

(Top) Tissue biomarker analysis was performed to quantify liver HBV+ hepatocytes by performing a mIF assay. A 4- plex assay was developed with HBsAg, HBV core, cell membrane (Na^+K^+-ATPase), and nuclear (DAPI/histone H3) markers using the Opal workflow (Akoya Biosciences). Briefly, formalin fixed, paraffin embedded (FFPE) tissues were sectioned at 5 µm thickness onto positively charged glass slides. Slides were baked for a minimum of 45 minutes in a 60°C dry oven, then loaded onto the Bond RX autostainer (Leica Biosystems). Slides underwent an automated dewaxing protocol comprised of treatment with Bond Dewax Solution (Leica Biosystems, AR9222) at 72°C

for 30 seconds and alcohol washes at ambient temperature. Heat induced epitope retrieval (HIER) protocol in the EDTA based pH 9 retrieval solution Bond Epitope Retrieval solution 2 (ER2, Leica AR9640) was performed for the unmasking of antigens at 100°C for 20 minutes. Hydrogen peroxidase block was applied at the start of each staining run to block endogenous peroxidase. Each round of antibody staining procedure commenced with the application of Antibody Diluent/Block (Akoya Biosciences ARD1001EA) for 10 minutes, followed by application of primary antibody for 60 minutes, then secondary antibody reagent Opal Polymer HRP Mouse + Rabbit (Akoya Biosciences ARH1001EA) for 10 minutes, then tyramide signal amplification (Opal Fluor Reagent Packs, Akoya Biosciences, FP1487001KT, FP1488001KT, FP1495001KT, FP1497001KT) for 10 minutes. Opal fluorophores were prepared by dissolving the lyophilized reagent into dimethyl sulfoxide, then diluting into 1X Plus Automation Amplification Diluent (Akoya Biosciences, FP1609) prior to run. A sufficient number of rinses with Bond Wash (Leica Biosystems), a buffer containing Tris buffered saline, surfactant and 3.5% ProClin with a pH of 7.5-7.7 at 25^{o}C (Leica Biosystems) was applied throughout the staining run as advised by the standard Akoya protocol. Antibodies were applied in the following order: position 1: HBcAg (HBV core antigen clone 366-2 Rabbit IgG, Gilead Sciences GS-1051053; 6 µg/mL; Opal Fluor 690); position 2: HBsAg (HBV surface antigen clone XTL17 Mouse IgG2a, Gilead Sciences GS-650225; 5 µg/mL; Opal Fluor 520) ; Histone H3 (nuclear marker clone EPR16987 Rabbit IgG, Abcam ab176842; working concentration 0.2 µg/mL; Opal Fluor 570); and Sodium Potassium ATPase (cell membrane marker clone EP1845Y Rabbit IgG, Abcam ab76020; 2 µg/mL; Opal Fluor 620). Immunofluorescent staining with Opal technology is

achieved when Opal fluorophores are successfully covalently bound in close vicinity to the epitopes on the tissue, followed by the primary antibody being cleaved with HIER (Ab strip). HBcAg and HBsAg were stripped from slides by undergoing HIER performed in the citrate-based pH 6 epitope retrieval solution Bond Epitope Retrieval solution 1 (ER1, Leica AR9961) at 95°C for 20 minutes. Histone H3 was stripped from slides by undergoing HIER performed in ER2 at 95°C for 40 minutes. Following the final round of antibody Spectral DAPI (Akoya Biosciences, FP1490) was applied to the slides for 5 minutes, then slides were rinsed and removed from the Bond RX autostainer. Slides were cover slipped with Prolong Gold Antifade mounting media (Thermo Fisher Scientific P36930) and glass coverslips (Dako Cover Glass, Agilent CS70430-2). Images were scanned on the Vectra Polaris (Akoya Biosciences). The finalized assay underwent testing for antibody stripping performance, optimal antibody order, and appropriate positive and negative controls. Histone H3 immunofluorescent staining was performed as a single plex assay to test for concordance of nuclei detection with spectral DAPI (Akoya Biosciences). In testing of clinical materials Histone H3 sufficiently detected nuclei where spectral DAPI failed. **(Bottom)** Peripheral biomarker analysis was performed on serum samples to quantify HBV RNA, HBsAg, HBcrAg, HBV DNA and ALT.

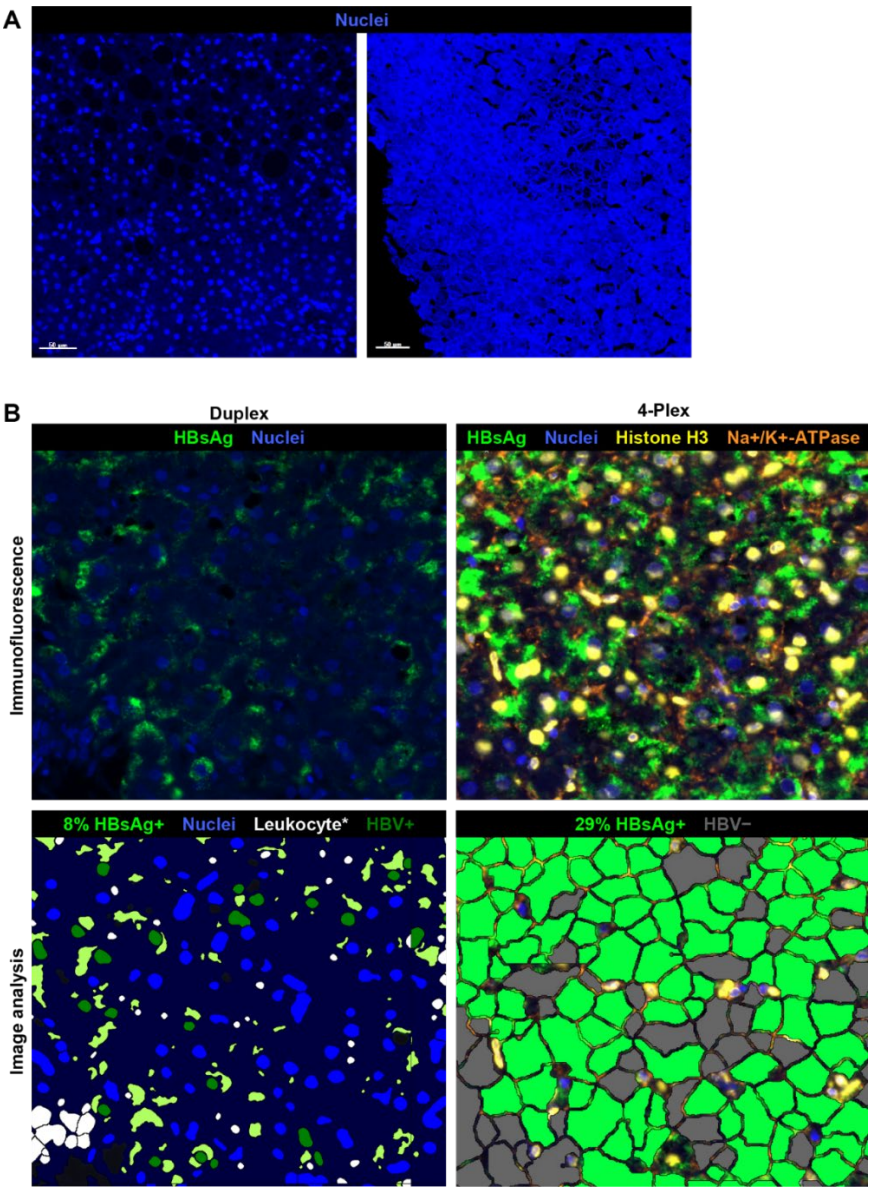

Fig. S3. Illustration of nuclear drop-out and area- vs cell- based assessment

(A) The right image is representative of nuclear dropout, where diffuse DAPI signal precludes nuclear segmentation **(B)** Top panel: IF images of a HBsAg+ sample stained as duplex (left) or 4-plex (right). Lower panel: Image analysis illustrates the challenge to associate positive HBsAg staining to a particular nucleus (left). *Size exclusion was used to classify 'Leukocytes'. The membrane stain allows a cell-based analysis and captures the %HBV+ hepatocytes accurately.

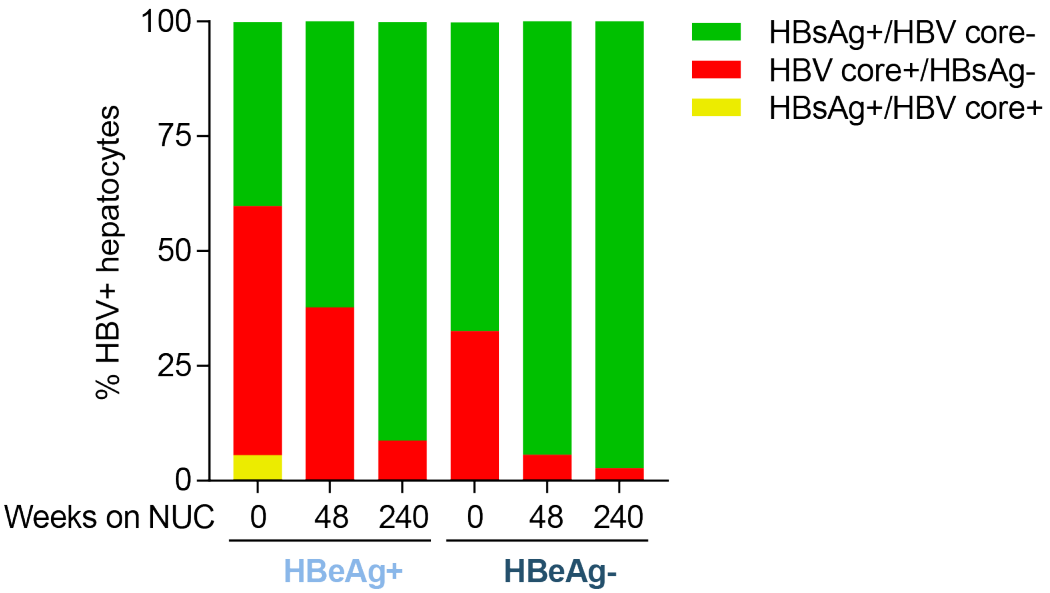

Fig. S4. Proportion of HBV+ hepatocytes that express HBsAg and/ or HBV core.

Proportion of total HBV+ hepatocytes that are HBsAg+ but HBV core- (green), HBV core+ but HBsAg- (red) or dual-positive (yellow) in HBeAg+ and HBeAg- patients before and after NUC treatment.

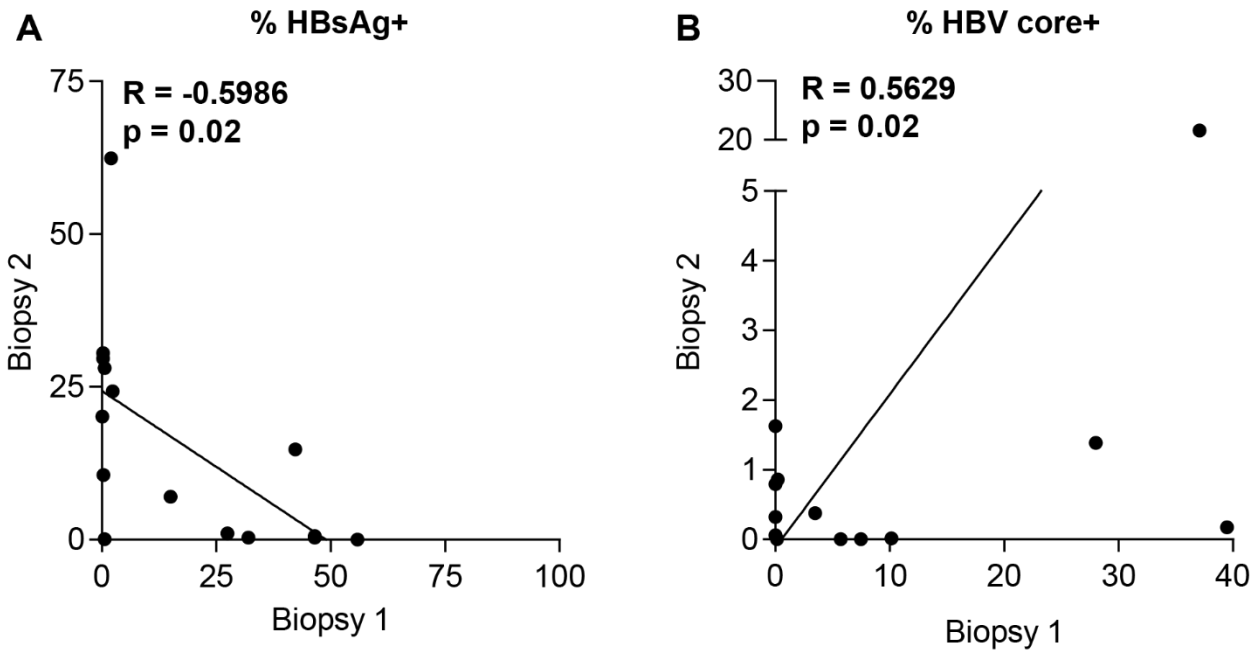

Fig. S5. Analysis of duplicate liver biopsies suggests inherent sampling heterogeneity for viral antigens.

Pearson's correlation analysis between HBsAg+ (A) and HBV core+ (B) hepatocytes in biopsy #1 vs. biopsy #2 from the same patient-timepoint.

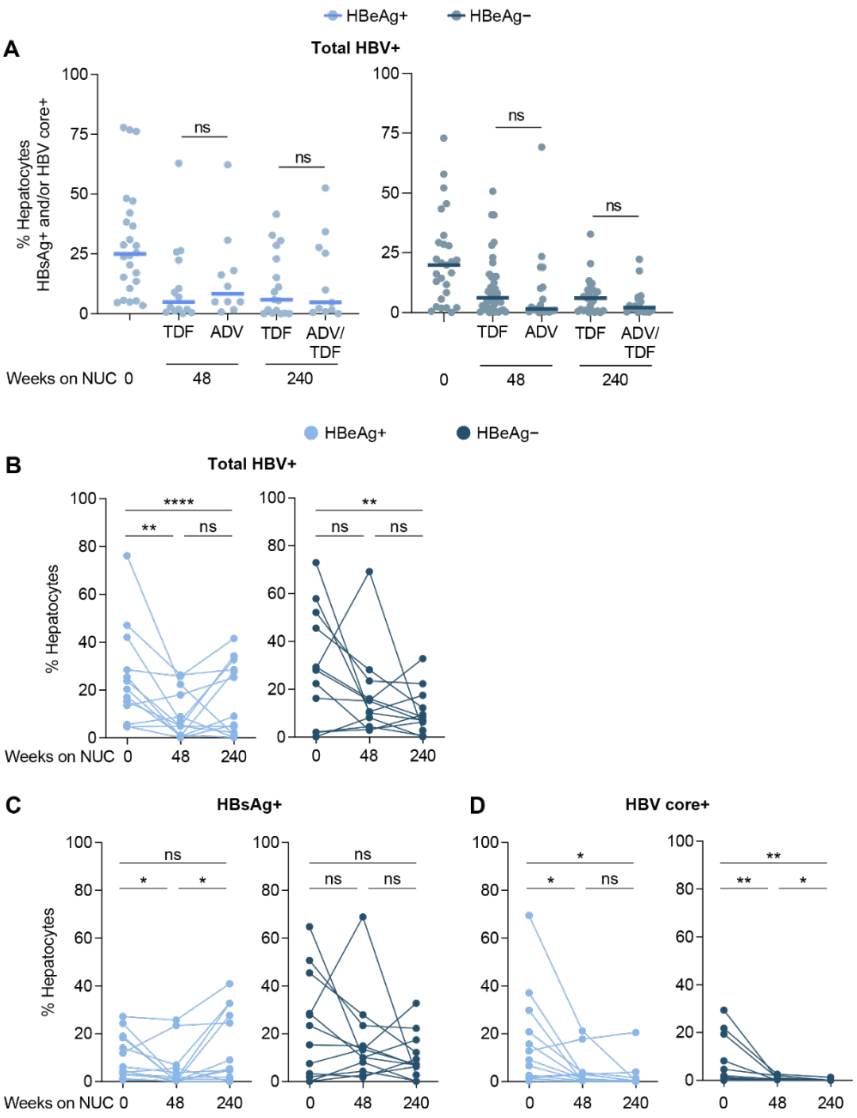

Fig. S6. Quantification of total HBV+ hepatocytes in CHB patients on NUC treatment.

(A) Quantification of total HBV+ hepatocytes in HBeAg+ (left, light blue) and HBeAg- (right, dark blue) subjects treated with TDF and/or ADV. Quantification of total HBV+ hepatocytes (B, defined as HBsAg+ and/or HBV core+), HBsAg+ hepatocytes (C), and HBV core+ hepatocytes (D) throughout NUC-treatment in a subset of HBeAg+ (light blue, left) and HBeAg- (dark blue, right) subjects. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ns = not significant, Mann-Whitney test (A), Wilcoxin test (B-D).

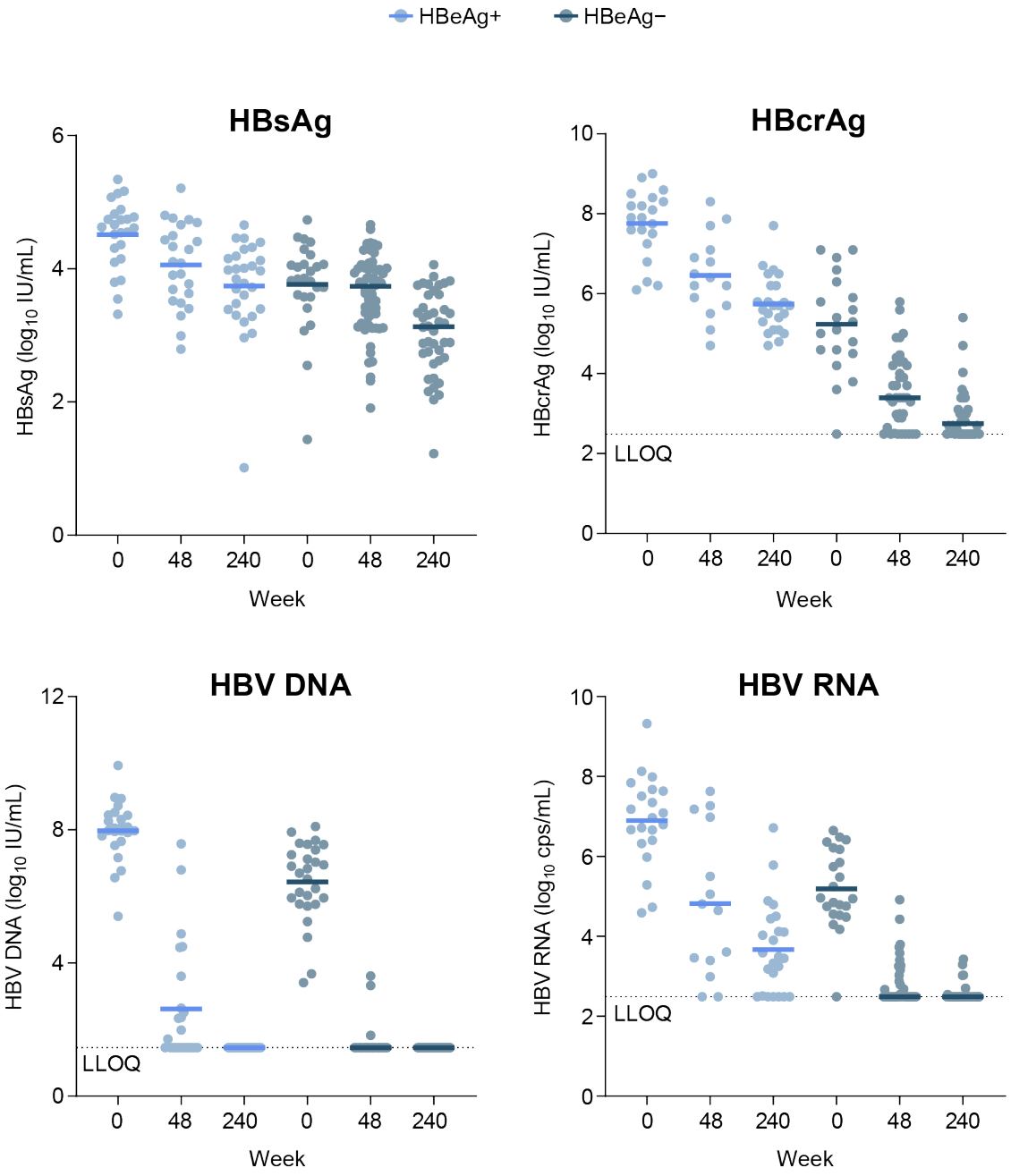

Fig. S7. Quantification of peripheral viral biomarkers in CHB patients on NUC treatment.

Quantification of peripheral viral biomarkers (HBsAg, HBcrAg, HBV DNA and HBV RNA) in HBeAg+ (light blue) and HBeAg- (dark blue) individuals throughout NUC treatment. LLOQ = lower limit of quantification

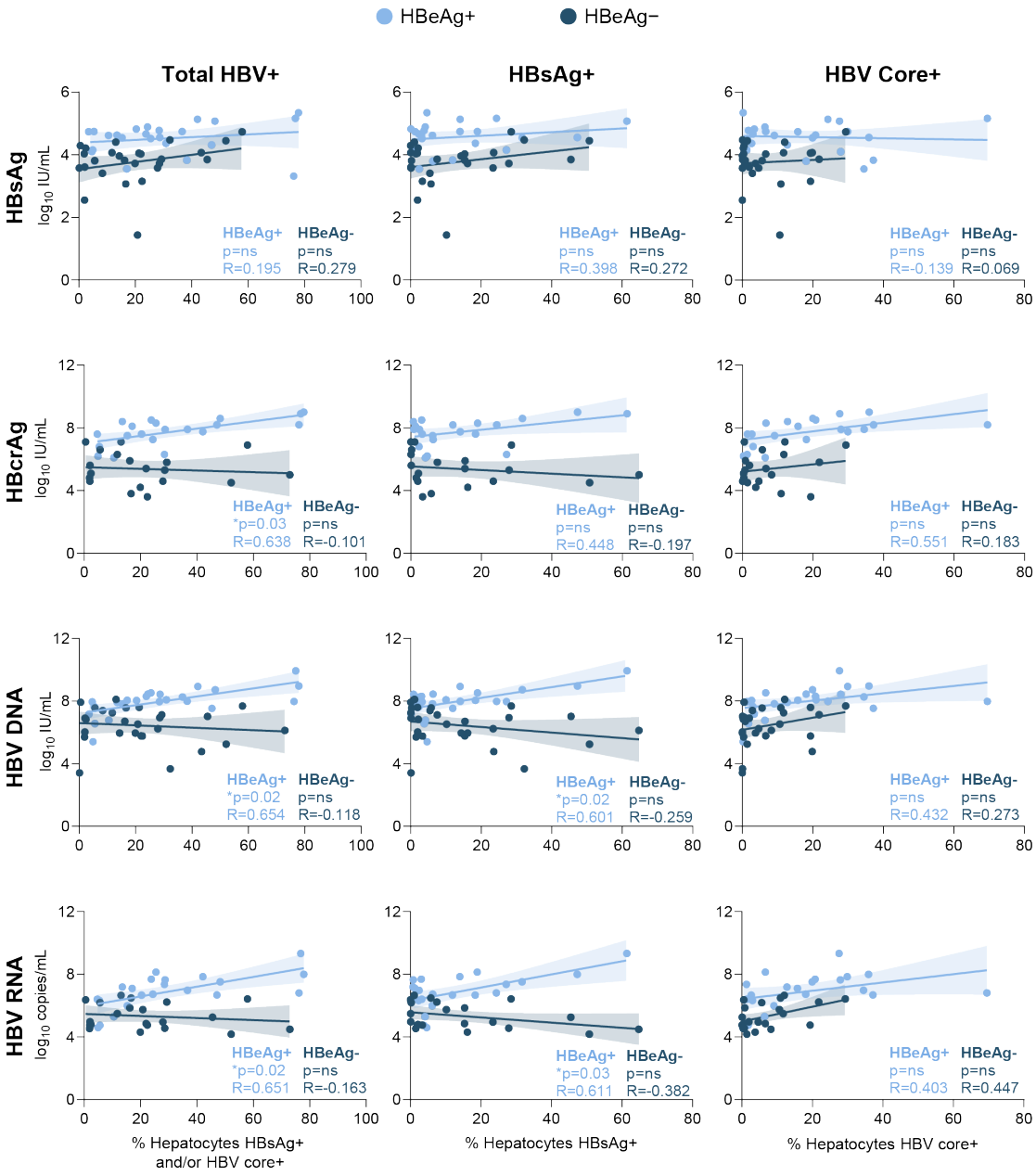

Fig. S8. Correlation between peripheral viral biomarkers and liver viral antigens at baseline

Correlations between peripheral viral biomarkers and total HBV+ hepatocytes (left), HBsAg+ hepatocytes (middle), or HBV core+ hepatocytes (right) in HBeAg+ (light blue) and HBeAg- (dark blue) patients at baseline. *Adjusted p<0.05: Pearson correlation analysis.

Table S1. Number of biopsies at each time point stratified by HBeAg status

	Baseline HBeAg Status		
Visits	**Negative**	**Positive**	**Total**
Baseline Only	0	1	1
BL+WK48	8	6	14
BL+WK48+WK240	12	13	25
BL+WK240	7	4	11
WK48+WK240	15	1	16
WK48 only	25	4	29
WK240 only	8	10	18
Total	75	39	114

Table S2. Correlation between peripheral viral biomarkers and HBsAg$^+$ hepatocytes

Correlation between peripheral viral biomarkers and HBsAg$^+$ hepatocytes										
HBeAg status	**Peripheral viral biomarker**	**Baseline**			**Week 48**			**Week 240**		
		R-value	**p-value**	**Adjusted p-value**	**R-value**	**p-value**	**Adjusted p-value**	**R-value**	**p-value**	**Adjusted p-value**
HBeAg$^+$	HBsAg	0.398	0.054	0.189	-0.084	0.696	0.835	0.288	0.137	0.355
	HBcrAg	0.448	0.047	0.174	-0.172	0.541	0.721	-0.127	0.556	0.721
	HBV DNA	**0.601**	**0.002**	***0.025**	-0.127	0.694	0.835	NR	—	—
	HBV RNA	**0.611**	**0.003**	***0.031**	-0.102	0.753	0.856	-0.229	0.36	0.629
HBeAg$^-$	HBsAg	0.272	0.18	0.382	0.12	0.362	0.628	**0.429**	**0.005**	***0.033**
	HBcrAg	-0.197	0.419	0.691	-0.143	0.442	0.694	-0.017	0.941	0.97
	HBV DNA	-0.259	0.192	0.395	NR	—	—	NR	—	—
	HBV RNA	-0.382	0.087	0.258	-0.453	0.09	0.258	0.075	0.888	0.96
Correlation between peripheral viral biomarkers and HBV core$^+$ hepatocytes										
HBeAg status	**Peripheral viral biomarker**	**Baseline**			**Week 48**			**Week 240**		
		R-value	**p-value**	**Adjusted p-value**	**R-value**	**p-value**	**Adjusted p-value**	**R-value**	**p-value**	**Adjusted p-value**
HBeAg$^+$	HBsAg	-0.139	0.517	0.629	0.269	0.204	0.396	-0.009	0.964	0.97
	HBcrAg	0.551	0.012	0.065	**0.663**	**0.007**	***0.047**	**0.653**	**0.001**	***0.018**
	HBV DNA	0.432	0.035	0.144	0.682	0.015	0.074	NR	—	—
	HBV RNA	0.403	0.07	0.232	0.592	0.043	0.165	**0.683**	**0.002**	***0.026**
HBeAg$^-$	HBsAg	0.069	0.739	0.856	-0.136	0.301	0.568	0.041	0.798	0.893
	HBcrAg	0.183	0.454	0.697	0.164	0.377	0.637	0.338	0.124	0.34
	HBV DNA	0.273	0.169	0.372	NR	—	—	NR	—	—
	HBV RNA	0.447	0.029	0.137	0.644	0.01	0.057	-0.668	0.147	0.354

*Adjusted p<0.05: Pearson correlation analysis, p-values adjusted for false discovery rate using Benjamini-Hochberg procedure. NR, not reported due to insufficient (<5) data points for analysis. Bold text indicates statistical significance with adjusted p-value. Correlation analyses with HBV core+ cells in NUC-treated subjects were based on very few positive HBV core+ cells, mostly driven by one outlier sample, as detected by mIF, so may be unreliable.