HBcrAg values may predict virologic and immunologic responses to pegIFN-α in NUC-suppressed HBeAg-negative chronic hepatitis B

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SUPPLEMENTARY MATERIALS AND METHODS

Patient populations. 53 HBeAg-negative patients with chronic hepatitis without any clinical evidence of cirrhosis (no US signs of portal hypertension or liver functionality reduction), under long-term NUC treatment and with complete viral suppression for at least 2-3 years were included in the present study. Patients were recruited from April 2015 and followed until March 2018. All patients provided written informed consent prior to enrolment. The study was approved by the local Ethic Committees at all participating sites and conducted in compliance with the Declaration of Helsinki, Good Clinical Practice guidelines, and local regulatory requirements.

Randomisation and Sample Size. The randomization was centralized at the Unit of Infectious Diseases and Hepatology in Parma (Coordinating Unit) and was stratified by individual participating centers at a 1:1 ratio. The randomization list was kept at the Coordinating Unit.
The sample size is typical for exploratory basic science studies, based on the analysis of highly complex immunological parameters, which cannot assess larger populations of patients because of feasibility constraints. No screening failure occurred.

Safety profile. Safety was evaluated by assessment of clinical laboratory tests (including measurements of serum HBsAg, anti-HBs, anti-HBe, HBV DNA), physical examinations, vital signs measurements, and by documentation of adverse events (AEs). All safety data were collected from the time of the first study drug dose to 30 days after the last dose.

Endpoints. The primary efficacy endpoint was the mean change in quantitative serum HBsAg (log10 IU/mL) from baseline to weeks (w) 24, 48, and 24PT. The secondary endpoints were i. the proportion of patients with HBsAg loss and seroconversion at weeks 24, 48 and 24PT, ii. the level of improvement of HBV-specific T cell responses at weeks 4, 12, 24 and 48 of pegIFN-α therapy and week 24PT compared to the control arm treated with NUC alone and iii. the level of correlation between decline in serum HBsAg and improvement of HBV-specific T cell responses. Additional endpoints were the assessment of possible serological (HBsAg and HBcrAg levels at baseline) or cellular immune (cytokine production and CD107 degranulations by HBV-specific CD4 and CD8 cells at baseline) baseline predictors of HBsAg decline induced by therapy.

HBV-specific T cell reactivity. HBV-specific T cell responses were studied longitudinally before, during and after pegIFN-α therapy following in vitro stimulation with overlapping peptides covering the overall HBV proteome. Responses of control NUC-suppressed patients who continued NUC therapy without pegIFN-α add-on were studied at the corresponding time points (figure 1A).

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To analyze global CD4+ and CD8+ reactivity against structural and non-structural HBV proteins, PBMC were stimulated with a panel of 315 15-mer peptides, overlapping by 10 residues, covering the overall HBV genotype D sequence, pooled in 8 mixtures (X, Core, Env 1, Env 2, Pol 1, Pol 2, Pol 3, Pol 4). A pool of immunodominant HLA-class I and II peptides from CMV, EBV, and influenza sequences served as controls. Immunological assays were performed on day 10 using anti-IFN-γ, anti-IL-2 (BD Biosciences) and anti-TNF-α (Miltenyi) conjugated mAbs for the detection of intracellular cytokines, and using an anti-CD107a antibody (BD Biosciences) for the study of the cytotoxic potential. Cells were acquired on FACSCANTO II and LSR Fortessa flow cytometers and were analyzed with the Flowjo software (BD Bioscences).

Cell surface staining and flow cytometry analysis. For ex vivo NK cell phenotypic analysis, PBMC were stained with the following fluorochrome-conjugated antibodies: CD16, CD56, CD3, 7AAD, CD94, HLA-DR, CD38, TRAIL (all from BD Biosciences); NKG2D (Biolegend); NKp30 and NKp46 (Miltenyi). To assess the proliferation capability, NK cells were permeabilized and stained with anti-Ki67 (BD Biosciences). Cells were acquired on FACSCANTO II and LSR Fortessa multicolour flow cytometers and were analyzed with the Flowjo software (BD Bioscences, San Jose, CA, USA).

Intracellular cytokine staining (ICS) and CD107a degranulation assay for NK cells. To measure IFN-γ and TNF-α production and CD107a degranulation by NK cells, intracellular staining was carried out on PBMC after overnight incubation in the presence or absence of IL-12 and IL-18 (10 ng/ml, R&D systems). PBMC were then incubated with or without K562 target cells (E:T ratio 5:1) and an anti-CD107a antibody (BD Biosciences) was added to cells treated with brefeldin-A (10 μg/ml, Sigma-Aldrich, UK) for additional 3 hours. After washing, cells were stained with anti-CD56, anti-CD3, anti-CD16 antibodies. Cells were fixed and permeabilized to allow intracellular detection of IFN-γ and TNF-α (Biolegend).

Statistical methods. Data were analyzed by the statistical packages GraphPad Prism (GraphPad Software, La Jolla, CA), R version 4.3.1 [R Core Team (2023). https://www.R-project.org/] with many additional packages, JASP version 0.17.3 (https://jasp-stats.org/) and Jamovi v. 2.4.5 (https://www.jamovi.org/).

The suitable cut-off for the different HBcrAg prognostic values at baseline was obtained using a ROC curve analysis, where HBsAg log decline (greater or less than 0.5 log) was used as classification criterion. The optimal threshold points on the ROC curves were chosen as those maximizing the difference between true positive and false-positive rates, the so-called Youden's

index. The Youden index is the farthest point on the ROC curve from the line of equality (diagonal line). AUC (area under the curve) value was equal to 0.88, showing very high discriminating power.

Multivariate analyses, in particular binary logistic regression and decision tree algorithms, were performed to assess the most relevant immune parameters allowing to distinguish different groups of pegIFN-α treated patients. Decision tree analysis was performed to evaluate the relevance of IFN-γ CD8 T-cell responses on HBsAg decline.

SUPPLEMENTARY RESULTS

The enhancement of antiviral T cell activity is multispecific and poly-functional. CD4- and CD8-mediated T cell responses targeting the polymerase region were found consistently more expressed at all time points (figure S2, grey bars). Importantly, in pegIFN-α treated-patients with greater log HBsAg decrease, a significant increase in cytokine production was sustained by polymerase, envelope and core at CD8 T cell level and by envelope and core at CD4 level (figure 3B and figure S2, blue bars). Instead, in patients with a lower HBsAg decline, CD4-mediated T-cell responses were poorly modulated by pegIFN-α therapy (with a more evident effect on polymerase- and envelope-induced responses), while virtually no effect was observed on HBV-specific CD8+ T-cells (figure 3B and figure S2, grey bars).

To understand how functionally broad was the improvement of HBV-specific T cell responses in patients receiving pegIFN-α treatment, we first analyzed changes in the percentage of double- and triple-positive CD4 and CD8 T cell before and after treatment. As shown in figure 4A, double-positive IFN-γ+TNF-α+ CD4 T cells were significantly increased by pegIFN-α treatment, showing fold increase values greater than 1 in 80% and 31% of patients with a HBsAg log decline greater and lower than 0.5, respectively (blue and grey bars and orange/yellow pie charts at the bottom). Moreover, pegIFN-α therapy significantly enhanced the frequencies of double IFN-γ+TNF-α+, IFN-γ+CD107a+ and TNF-α+CD107a CD8 T cells, as well as of triple IFN-γ+TNF-α+CD107a+ CD8 T cells in patients with >0.5 log HBsAg reductions. This effect was not observed in patients with log reductions lower than 0.5 (figure 4B, blue and grey bars and circles, respectively). In particular, 70%, 90%, 90% and 70% of the patients with better serum HBsAg decline showed fold changes greater than 1 in IFN-γ+TNF-α+, IFN-γ+CD107a+, TNF-α+CD107a and IFN-γ+TNF-α+CD107a+ CD8 T cells (figure 4B, orange/yellow pie charts at the bottom).

Finally, we assessed how many of the tested CD4 and CD8 T-cell functions (IFN-γ, TNF-α, IL-2 production and CD107a degranulation) were simultaneously improved in individual subjects

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comparing week 24PT to baseline. Globally, in patients receiving pegIFN-α therapy a multifunctional CD4 and CD8 T cell improvement (≥ 3 functions) was detected in 45% and in 46% of the patients, respectively (figure 4C, left panel), with a broader recovery of CD4 and CD8 responses in patients with a higher HBsAg decline compared to patients with a log decline <0.5 (restoration of ≥ 3 functions: 64% vs 27% among CD4 T cells; 73% vs 18% among CD8 T cells; figure 4C, middle and right pies).

SUPPLEMENTARY FIGURES

Supplementary figure 1. Kinetics of HBsAg. A) Changes in HBsAg levels in NUC/PegIFN-α treated patients with HBsAg Log10 decline < 0.5 (grey bars on the left) and > 0.5 (blue bars on the right) from baseline to week 48 post-treatment. **B)** Changes in HBsAg levels in NUC treated patients. Each dot indicates an individual patient and each column shows the mean of HBsAg at the indicated time points.

Supplementary figure 2. Longitudinal evaluation of HBV-specific CD4 and CD8 T cell reactivity during and after pegIFN-α therapy. Cytokine production and ability to degranulate by HBV-specific CD4 and CD8 T cells were analyzed after 10 days in vitro stimulation with HBV peptide pools. **A)** Ratio (fold increase) between IFN-γ (top), TNF-α (middle) and IL-2 (bottom) production by HBV-specific CD4 T cells detected at the indicated time points compared to baseline from patients receiving pegylated interferon (with HBsAg log reduction lower or greater than 0.5; grey and blue bars, respectively), and from the NUC treated group (white bars). Each column shows the median with interquartile interval (25th-75th percentile) of the fold increase of each patient population at the indicated time points. The horizontal dotted line corresponds to fold increase value of 1. **B)** Effect of pegIFN-α therapy on IFN-γ, TNF-α and IL-2 production by HBV-specific CD8 T cells as well as the CD107 degranulation capacity, illustrated as in panel A. Statistics by the Wilcoxon-signed-rank test.

Supplementary figure 3. Antigen-specificity of HBV-specific T cells. The bars indicate the mean percentage of CD4 and CD8 T cells able to produce IFN-γ, TNF-α, IL-2 production and to degranulate in response to each viral antigen at baseline and W24PT in pegIFN-α treated patients belonging to the different sub-groups with HBsAg Log decline > or < 0.5. Statistics by the Wilcoxon-matched-paired test.

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SUPPLEMENTARY TABLES

Supplementary Table 1

	Patient ID	HBsAg (UI/mL)						
		Baseline	Week 4	Week 12	Week 24	Week 48	Week 24PT	Week 48PT
NUC/pegIFN-α treated patients	BOA-001	104.73	128.26	119.7	-	-	52.5	55
	BOA-002	852.95	1187.58	818.3	235.86	241.08	375.81	-
	BOA-004	408.69	433.44	189.32	142.56	-	61.92	64
	BOA-005	167.27	165.61	133.28	146.84	-	125.74	-
	BOV-001	5887.26	7937.16	6552.28	4489.05	4125.85	2509.87	-
	BOV-002	1204.17	2195.2	1698.98	616.17	456.47	212.49	-
	BOV-003	214.92	569.96	498.85	226.1	399.91	143.42	-
	BOV-004	444.07	230.54	12.9	0	-	0	-
	MI-001	372.7	478.7	549.4	540	-	327	326
	MI-002	446.7	515.1	655.2	577.8	-	327	323
	MI-003	259.7	370.6	82	0.39	0.06	0.1	0.2
	MI-004	118.4	146.4	129.1	-	-	110	110
	MI-005	2913	4067	4777	3807	-	2507	2259
	MI-006	480.4	590.2	586.6	364.4	295	196.7	200
	PI-001	1378	1066.8	1474	1274.88	884.74	876.71	639.75
	PI-002	562	659	427	205	120.07	84.2	3.19
	PI-004	1483.75	1920	2502	478.56	73	452.3	620.34
	PI-007	1116	1284	1004	512	403	79.59	50.79
	PR-002	998.34	-	1152.09	950.67	-	488	-
	PR-003	280.51	-	387.9	455.17	-	137	-
	PR-004	841.05	-	963.08	909.87	-	496	-
	PR-005	2285.55	-	2094.59	1573.81	-	1199	-
	PR-006	675.03	-	637.52	539.33	-	166	-
	PR-012	2382.72	-	2962.43	2379.77	-	1405	-
	RE-001	1759.51	2034.57	1188.22	177.22	241.36	212.61	189.65
	RE-002	910.34	1032.06	882.25	737.19	730.93	628.42	464.85
	RE-003	217.92	413	238.73	4.15	9.62	7.13	4.51
	RE-004	740	760.99	752.41	492.43	231	135.64	204.52
	RE-005	2891.98	2883.81	2592.31	1890.95	854.07	206.46	236.3
NUC treated patients	BOA-003	2134.36	-	2247.52	2895.85	2163.22	2003.58	-
	BOA-006	3852.03	-	3006.75	3593.87	3041.15	2880.94	3411
	BOV-005	2057.03	2010.72	-	-	-	-	-
	BOV-006	8720.48	8353.38	8107.61	7489.43	8349.7	6940.23	-
	BOV-007	626.41	626.75	506.76	489.75	454.12	431.12	-
	MI-007	480.2	468.7	452.4	407.3	440.5	394.1	-
	MI-008	783.4	761	704.9	755.7	647.4	580.5	-
	MI-009	143.1	138.3	140.4	135.9	125.8	117.9	-
	MI-010	3964	3517	3257	3054	3027	2978	-
	MI-011	1900	1685	1635	1500	1407	1521	-
	MI-012	1859	1549	1503	1150	1124	964	-
	PI-003	3558.1	2950	3426	3609	2583	2995.98	-
	PI-005	499.59	507	527	509	385	422	282.05
	PI-006	2118	1802	2783	2240	1779	1943.26	1443.3
	PI-008	1704	-	1676	1561	1305	1604.97	1216.15
	PR-001	808.42	-	-	902.8	-	760.93	-
	PR-007	6529.75	-	5826.69	6006.89	-	5604	-
	PR-008	746.14	-	649.66	651.65	-	454.11	-
	PR-009	1153.56	-	1245.56	1166.01	-	1081.55	-
	PR-010	1566.46	-	-	1784.18	-	-	1581.55
	PR-011	681.23	-	-	625.11	-	-	-
	RE-007	949	787.07	671.33	877.8	956.61	829.78	785.27
	RE-008	1184.23	1060.35	1143.65	881.15	1035.98	1054.2	1096.34
	RE-009	169.32	128.66	92.88	64.14	50.96	28.56	16.02

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Supplementary Table 2

	Patient ID	HBcrAg (LogU/mL)	
		Baseline	Week 24PT
NUC/pegIFN-α treated patients	**BOA-001**	<2.5	<2.5
	BOA-002	3.4	<2.5
	BOA-004	<2.5	2.5
	BOA-005	3.1	2.8
	BOV-001	4.8	4.5
	BOV-002	<2.5	<2.5
	BOV-003	4	<2.5
	BOV-004	<2.5	3.6
	MI-001	3.1	4
	MI-002	2.5	3.3
	MI-003	<2.5	<2.5
	MI-004	4.5	4.3
	MI-005	3.3	3.1
	MI-006	3.3	2.7
	PI-001	2.5	-
	PI-002	<2.5	<2.5
	PI-004	2.5	<2.5
	PI-007	<2.5	<2.5
	PR-002	<2.5	<2.5
	PR-003	<2.5	-
	PR-004	5.6	5.3
	PR-005	<2.5	-
	PR-006	<2.5	<2.5
	PR-012	<2.5	-
	RE-001	3.3	3
	RE-002	4.1	4.1
	RE-003	<2.5	<2.5
	RE-004	2.9	2.7
	RE-005	3	<2.5
NUC treated patients	**BOA-003**	3.4	3.2
	BOA-006	<2.5	<2.5
	BOV-005	-	-
	BOV-006	<2.5	-
	BOV-007	-	<2.5
	MI-007	<2.5	<2.5
	MI-008	4.3	4.2
	MI-009	<2.5	-
	MI-010	3.8	3.7
	MI-011	3	2.9
	MI-012	4	3.8
	PI-003	3.3	3.3
	PI-005	<2.5	<2.5
	PI-006	<2.5	<2.5
	PI-008	3.8	3.9
	PR-001	<2.5	<2.5
	PR-007	3.2	3.1
	PR-008	<2.5	2.9
	PR-009	<2.5	<2.5
	PR-010	3.4	3.4
	PR-011	2.6	-
	RE-007	<2.5	<2.5
	RE-008	<2.5	<2.5
	RE-009	<2.5	2.5

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