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Viral Hepatitis

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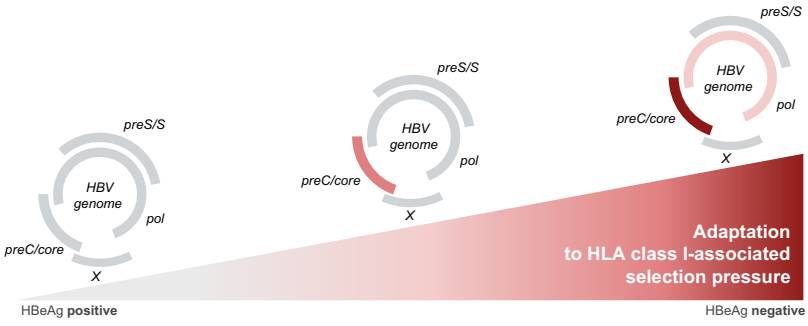
Graphical abstract



Highlights

- 532 complete viral genomes from people with chronic HBV infection were sequenced.
- HLA-associated mutational states (HAMs) indicative for CD8 T cell pressure were identified.
- HAMs are more frequent in HBV core compared to other HBV proteins.
- HBV genomes show different levels of adaptation between patients and viral proteins.
- The level of adaptation of HBV to CD8 T cell pressure correlates with markers of replication.

Impact and implications

The immune response mediated by CD8 T cells plays a critical role in controlling HBV infection and shows promise for therapeutic strategies aimed at achieving a functional cure. This study demonstrates that mutational escape within CD8 T-cell epitopes is common in HBV and represents a key factor in the failure of immune control. Notably, the HBV core protein emerges as the primary target of CD8 T-cell selection pressure. Additionally, the observed correlation between HBV adaptation levels and viral replication markers indicates that CD8 T-cell immunity may influence transitions between phases of chronic HBV infection.

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HBV shows different levels of adaptation to HLA class I-associated selection pressure correlating with markers of replication

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Background & Aims: Immune responses by CD8 T cells are essential for control of HBV replication. Although selection of escape mutations in CD8 T-cell epitopes has previously been described in HBV infection, its overall influence on HBV sequence diversity and correlation with markers of HBV replication remain unclear.

Methods: Whole-genome sequencing was applied to HBV isolates from 532 patients with chronic HBV infection and high-resolution HLA class I genotyping. Using a Bayesian model (HAMdetector) for identification of HLA-associated mutational states (HAMs), the frequency and location of residues under CD8 T-cell selection pressure were determined and the levels of adaptation of individual isolates were quantified.

Results: Using previously published thresholds for the identification of HAMs, a total of 295 residues showed evidence of CD8 T-cell escape, the majority of which were located in previously unidentified epitopes. Interestingly, HAMs were highly enriched in the HBV core protein compared to all other proteins. When individual HBV isolates were compared, different levels of adaptation to HLA class I immune pressure were noted. The level of adaptation increased with patient age and correlated with markers of replication, with low levels of adaptation in HBeAg-positive infection. Furthermore, the levels of adaptation negatively correlated with HBV viral load and HBsAg levels, consistent with high levels of HLA class I-associated selection pressure in patients with low replication levels.

Conclusions: HBV sequence diversity is shaped by HLA class I-associated selection pressure with the HBV core protein being a predominant target of selection. Importantly, different levels of adaptation to immune pressure were observed between HBV infection stages, which need to be considered in the context of T-cell-based therapies.

Introduction

HBV infection remains a significant global health concern, with over 250 million individuals estimated to be chronically infected worldwide.[1] HBV is a small, enveloped DNA virus belonging to the Hepadnaviridae family that primarily targets the liver, leading to a wide spectrum of clinical outcomes, ranging from asymptomatic carriers to severe liver disease, including cirrhosis and hepatocellular carcinoma.[2] The hepatitis B e antigen (HBeAg) is an important marker for classification of the different clinical stages of HBV infection. Although not required for replication, there is evidence that HBeAg is involved in the establishment of chronic infection and functions as an immune tolerogen (for a review, see[3]). Loss of HBeAg is typically associated with a flare of hepatitis, activation of CD8 T cells[4] and reduced levels of replication. However, in some patients, high-level replication of HBeAg-negative viral variants persists.[2]

Immune responses by HBV-specific CD8 T cells play a central role in determining the outcome of infection.[5] CD8 T cells contribute to controlling HBV infection but may also contribute to liver pathology during chronic hepatitis B.[6] CD8 T cells target HBV-infected hepatocytes via interactions of their T-cell receptor (TCR) with specific viral epitopes presented on the surface of infected cells in the context of the HLA class I complex. Failure of the CD8 T-cell response against HBV in the context of chronic

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infection has been described and includes a progressive dysfunction of HBV-specific CD8 T cells upon continuous antigen stimulation in the liver, also termed T-cell exhaustion.[7,8] In addition, selection of viral epitope variants associated with immune escape also contributes to CD8 T-cell failure in HBV infection.[9] Selected mutations in HBV epitopes can impair binding to the HLA class I molecule or alter the interaction of the TCR with the variant epitope in the HLA-complex.

Understanding the extent and the mechanisms underlying the selection of escape mutations within CD8 T-cell epitopes is of paramount importance in the context of both natural HBV infection and therapeutic interventions, such as immunotherapies. In HBV, the existing data on CD8 T-cell escape is correlative, showing that substitutions are enriched in targeted epitopes during chronic infection and functionally impair the CD8 T-cell response.[10–14] In prior HLA class I-association studies, these substitutions have been observed to be statistically more frequent in patients carrying the relevant HLA class I alleles consistent with their specific selection.[13] Notably, data on CD8 T-cell escape in HBV has only been obtained for individual immunodominant epitopes or substitution patterns in the HBV core protein.[10–13,15] The full extent of HLA class I-associated mutations across all HBV proteins and the different levels of adaptation of individual HBV isolates is therefore unknown.

Herein, we combined full HBV genome sequence data from 532 patients with chronic infection with high-resolution HLA class I genotyping and applied novel tools for detection of HLA-associated mutational states (HAMs) in the viral genome to get a comprehensive map of the frequency and distribution of substitutions selected by HLA class I-restricted CD8 T cells. The results show that selection pressure is predominantly exerted on epitopes in the HBV core protein. Moreover, different frequencies of HAMs were noted for individual viral genomes correlating with markers of viral replication, such as the HBeAg status and HBV DNA concentrations in serum. Collectively, the data demonstrate extensive but also different levels of adaptation of viral genomes to CD8 T-cell pressure in chronic hepatitis B. Different levels of adaptation may influence the perspective for functional cure of hepatitis B, especially when T-cell-based therapeutic concepts are applied.

Patients and methods

Patients

In a multicenter effort, samples from 532 patients with HBV were collected at the Universities of Bonn, Düsseldorf, Essen, Freiburg, Hamburg, Hannover (all Germany) and London (UK) (Table 1 and Table S1). Informed consent was obtained from each patient, and the study protocol was approved by the local ethics committee of the Medical Faculty of Düsseldorf in accordance with the guidelines of the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation (Biocoll; Biochrom).[13] DNA for HLA-typing was extracted from PBMCs using spin columns (Qiagen). High-resolution (second field) HLA-A and HLA-B typing was performed by use of sequence-specific oligonucleotides (LABType™) methodology,[16] provided by One Lambda (Thermo Fisher Inc.).

Table 1. Patient characteristics.

	n (%)
Total, N	532 (100)
Sex	
Male	292 (55)
Female	224 (42)
No data	28 (5)
Age (years)	
Median	40
Range	18-85
Genotype	
A	101 (19)
B	27 (5)
C	13 (2)
D	372 (70)
E	20 (4)
Viral load (IU/ml)	
Median	4,494
IQR	1,294-94,846
HBsAg (IU/ml)	
Median	6,304
IQR	1,316-11,031
HBeAg status	
Positive	58
Negative	350
No data	124

HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen.

Amplification and sequence analysis of the HBV genome

Viral nucleic acid from 400 μl plasma was extracted automatically using the EZ1 Virus Mini Kit v2.0 on an EZ1 Advanced XL robot or manually (both Qiagen) and the complete HBV genome was amplified in two fragments as previously described.[17,18] In brief, two-step nested PCRs were performed for the core region (nucleotide [nt] 1,683-nt 2,399; 717 base pairs [bp] according to the reference genome NC_003977.2) and the polymerase region (nt 2,299-nt 1,798; 2,682 bp according to the reference genome NC_003977.2) with primers listed in Table S2. Amplicons were Sanger sequenced with primers listed in Table S2. Sequences were aligned with the software Geneious 10.2.6 (RRID:SCR_010519). Sequences were submitted to GenBank and are available under accession numbers (MZ043025-MZ043097; MZ097624-MZ097884).

Phylogenetic analysis of viral sequences and HAMdetector analysis

To analyze the phylogeny and to provide the input files for the HAMdetector tool, all obtained sequences were aligned with the software Geneious 10.2.6 (RRID:SCR_010519) using MAFFT.[19] For phylogenetic analysis, a tree based on the complete HBV sequence, with references from Genebank, was calculated in Geneious 10.2.8 with the Mr. Bayes plugin.[20] For visualization, the output was exported as Newick file with support values and visualized with iTol.[21] HAMdetector[22] is implemented as a julia package for identifying HLA-associated substitutions based on aligned viral sequences paired to host HLA class I data. It integrates information from epitope prediction via MHCflurry 2.0 and phylogeny (based on RAxML-NG). The model is fit using Stan and the complete source code and documentation is available at https://github.com/HAMdetector/Escape.jl. For prediction, the open-reading frames for precore, polymerase (pol), L-HBsAg (HBs) and the

x protein (HBx) were inferred from the HBV alignment used above and were translated into an amino acid sequence. No adjustments were made to sequences where the amber-stop codon at position 27 in precore was the majority or sequences with insertion or deletions in the pol/preS1 region. Therefore, the numbering of amino acids in the HAM detector output is not consistent with IEDB (Immune Epitope Database) or GenBank numbering. The final amino acid alignment, including numbering, can be found in the supplementary material. For HAMdetector phylogeny, the MAFFT nucleotide alignment used for phylogenetic analysis was used. The complete output data and sequence alignments are available as described in the data availability statement.

Analysis of HAMs in previously published epitopes

Previously published HBV epitopes were retrieved from the IEDB. Parameters for the database search were as follows: linear peptides, organism hepatitis B virus (ID 10407), host human, assay T-cell with positive result, MHC restriction class I. A list of 310 entries was generated, which was further manually edited to remove epitopes with an undefined restricting HLA class I type, insufficiently mapped epitopes (length ≥13 amino acids) and duplicate entries of different variants of the same epitope. The final list contained 141 entries (42 in precore/core, 54 in pol, 34 in HBsAg and 11 in the HBx protein). To provide evidence for HLA class I-associated selection pressure on residues within the epitopes, the maximum posterior probabilities for the relevant HLA class I types were determined using HAMdetector for all positions in the epitope.

Quantification of HLA class I adaptation of individual HBV isolates

The level of adaptation of the HBV polyprotein to HLA class I-associated selection pressure was quantified. For this purpose, a majority consensus sequence was generated for each HBV genotype and all isolates of the corresponding genotype were aligned to the consensus sequence. Only positions that differed from the consensus sequence were included in the subsequent calculation of an adaptation score. For these variant amino acids, the maximum posterior probabilities for all relevant HLA class I alleles of the individual patient were determined and subtracted by 0.5, as only values >0.5 are indicative of HAMs. The adaptation score was then calculated as the sum of the maximum posterior probabilities for all variant positions after subtraction of 0.5.

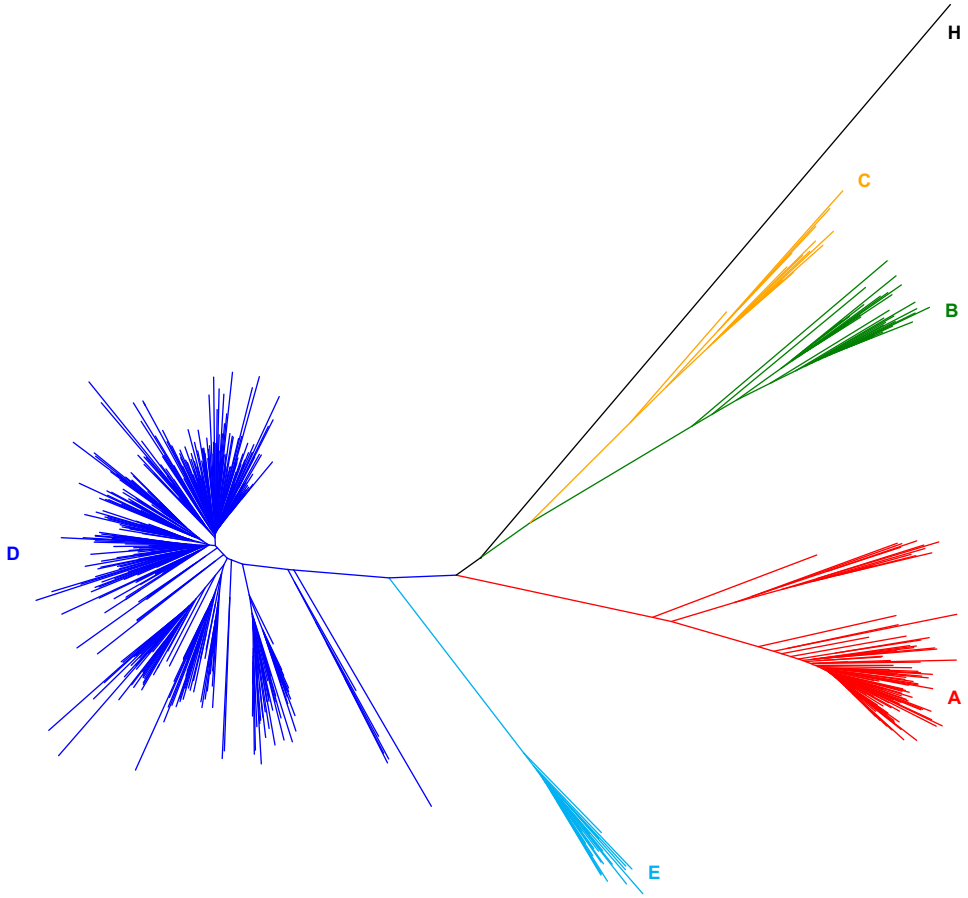

Fig. 1. Phylogenetic tree of all HBV sequences of the multicenter cohort. A total of 532 complete HBV sequences were aligned with genotype reference sequences [from ICTV] using MAFFT. A phylogenetic tree was calculated with the Mr. Bayes Plugin[20] in the software *Geneious 10.2.8* using the GTR genetic distance model and genotype H as outgroup. Genotypes are color coded (A-H) as indicated.

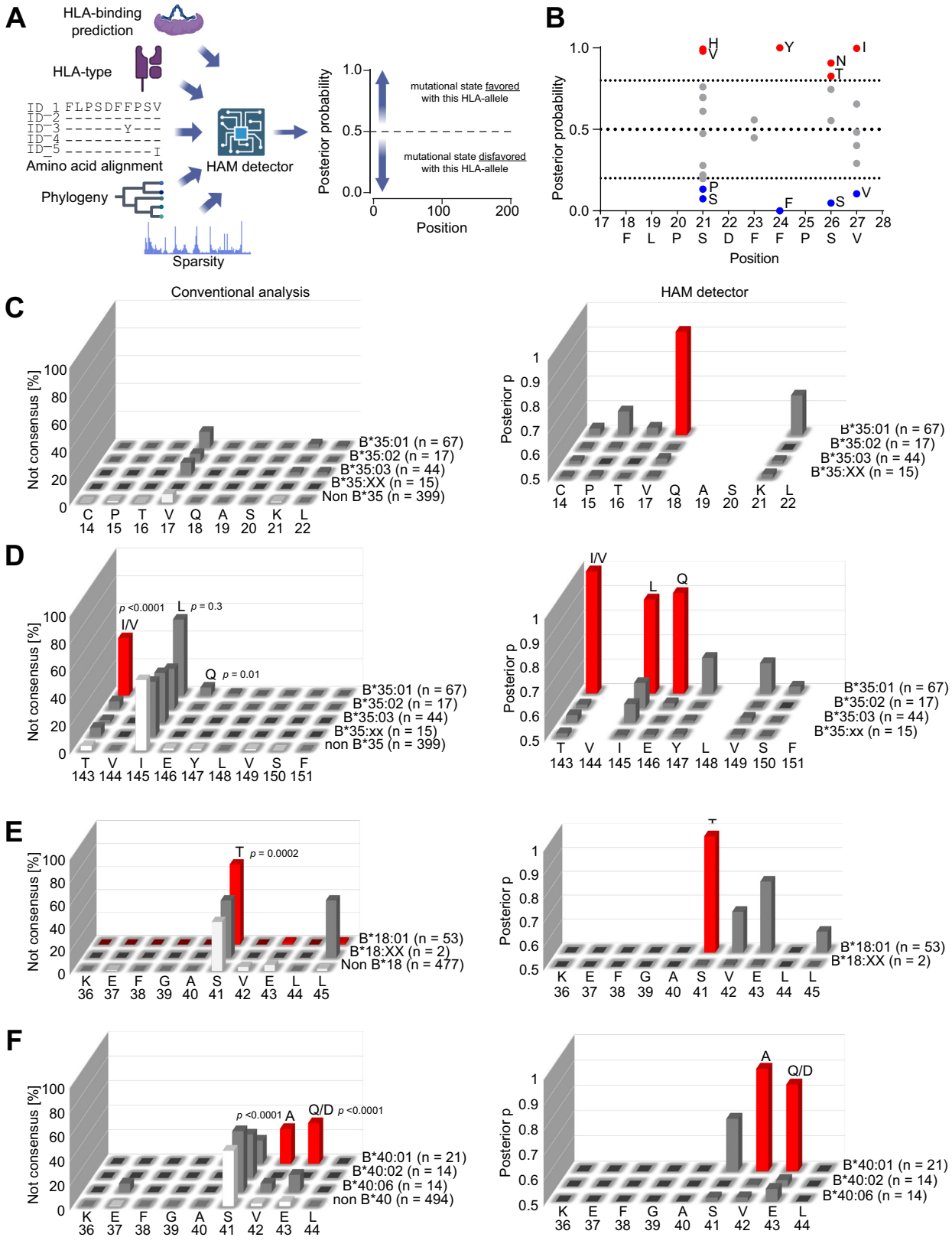

Fig. 2. HAMdetector and conventional statistical analysis for identification of HLA class I-associated viral sequence polymorphisms. (A) Schematic illustration of the input and output information of HAMdetector. The model integrates the alignment of viral sequences, the HLA alleles of patients, sparsity of HLA substitution associations, the phylogeny of viral sequences and potential HLA class I-binding motifs and assigns a posterior probability to all amino acids in the alignment. High posterior probabilities indicate that the amino acid is favored in individuals with the HLA allele, low posterior probabilities indicate that the amino acid is disfavored in

Results

Identification of residues under selection pressure with the HAMdetector tool

The full viral genome from 532 patients with chronic HBV infection was amplified in two fragments and sequenced. For genotyping, a phylogenetic tree with all viral sequences from the study as well as reference sequences from the NCBI was constructed (Fig. 1). The majority of the viral sequences from the cohort were genotype D (n = 372; 69.9%) and genotype A (n = 100; 18.8%). As expected for a European cohort, other genotypes such as genotype B (n = 27; 5.0%), genotype C (n = 13; 2.4%) and genotype E (n = 20; 3.7%) were less frequent (Table 1). The HLA class I-genotype was determined for all patients, allowing for association studies with viral sequence features. The complete patient characteristics are shown in Table S1.

For detection of residues under HLA class I-associated selection pressure (HAMs) in HBV, we utilized the HAMdetector tool.[22] The tool comprises a unified Bayesian regression model that combines multiple sources of information. These integrated factors include the alignment of viral sequences, patients' HLA alleles, sparsity of HLA substitution associations, the phylogeny of viral sequences and potential HLA class I-binding motifs (Fig. 2A). The outcome of this integration is a set of posterior probabilities for HLA substitution associations, which range from 0 to 1. The majority of posterior probabilities are close to 0.5, indicating that the amino acid is neither favored nor disfavored in the presence of the HLA class I allele. High posterior probabilities with values close to 1 indicate that the amino acid is favored in individuals with this HLA class I allele consistent with CD8 T-cell escape. Low posterior probabilities indicate that the amino acid is disfavored in the presence of this allele, suggesting that this amino acid is not a CD8 T-cell escape mutation. In Fig. 2B the results are illustrated for the well-described epitope $core_{18-27}$. For example, in this epitope, the F24Y substitution is a described immune escape variant.[17] Phenylalanine (F) at position 24 has a posterior probability of 1 for being a favored mutational state in HLA-A*02-positive patients. Conversely, the amino acid tyrosine (Y) at the same position has a posterior probability of 0, indicating it is a highly disfavored mutational state in patients with HLA-A*02.

Fig. 2C–F presents conventional analyses of substitution frequencies for three candidate epitope regions, compared to results obtained from HAMdetector. Traditional statistical methods for identifying "HLA footprints" involve creating a 2x2 table that counts the number of viral sequences with and without a substitution and with or without a specific HLA allele, followed by a statistical test, such as Fisher's exact test. The results of Fisher's exact test for associations identified by HAMdetector are shown in Fig. 2. Many HAMs identified by HAMdetector were also recognized as statistically significant in conventional analyses, although there are exceptions. For instance, for the candidate epitope (pol_{14-22} CPTVKASKL), there was a high posterior probability for phenylalanine at position 4 of the epitope (V17F) as the favored amino acid in HLA-B*35:01-positive individuals. In conventional analysis, a p value of 0.11 would not support immune selection of the V17F substitution. The advantage of HAMdetector lies in its inclusion of additional relevant information. In the model, a substitution that is "unexpected" at a certain position based on phylogeny is more likely to result from selection pressure. Conversely, polymorphisms typical of distinct genotypes or phylogenetic clades are less likely to be the result of selection pressure. Another crucial factor is sparsity. Comprehensive epitope maps show that HLA epitopes often overlap. Statistically, this means an association between a substitution and one HLA allele suggests that associations with other HLA alleles are also more likely. Additionally, relevant HLA class I motif information is included. HAMdetector considers substitutions in regions matching the binding motif of the relevant HLA class I type more likely to be HAMs. Based on this additional information, the algorithm assigns a high posterior probability to the V17F substitution as a HAM. Notably, the candidate epitope pol_{14-22} CPTVKASKL was experimentally confirmed in immunological assays (a representative result is shown in Fig. S1).

In a second HLA-B*35:01-restricted candidate epitope identified in the core region ($core_{143-151}$ TVIEYLVSF; Fig. 2D), HAMs were detected in three positions. Conventional analysis, however, indicated CD8 T-cell escape at only one position. Notably, HAMdetector identified a putative escape residue at the highly polymorphic position three of the epitope, with leucine being favored at this position in HLA-B35:01-positive individuals. We were able to experimentally confirm this novel candidate epitope in HLA-B*35-positive individuals and also tested peptide variants with the putative escape mutations (Fig. S1). The results further support that the substitutions functionally act as immune escape mutations.

Finally, in a third candidate epitope in the core ($core_{36-44}$ KEFGASVEL), HAMdetector identified HAMs in the context of HLA-B*18:01 (Fig. 2E) and distinct HAMs in the same epitope region in the context of HLA-B*40:01 (Fig. 2F). In this case, the HAMs would also have been identified by conventional statistical analysis. The candidate epitope was experimentally confirmed in the context of HLA-B*18:01 (Fig. S1). In conclusion, the results demonstrate that the HAMdetector is a powerful tool for the identification of HLA class I-associated viral sequence polymorphisms and allows for the identification of novel CD8+ T-cell epitopes.

The majority of HAMs are located in the HBV precore/core protein

Multiple sequence alignments were created for precore/core, pol, large HBsAg and HBx for identification of HAMs in all HBV

individuals with the HLA allele. A posterior probability of 0.5 indicates that the amino acid is neither favored nor disfavored. (B-D) Comparison of conventional statistical analysis (left column) with HAMdetector results (right column) for different candidate epitope regions and HLA class I genotypes. (B) Illustration of HAMdetector scores for the HLA-A*02-restricted epitope $core_{18-27}$. (C) HLA-B*35:01 pol_{14-22} CPTVKASKL (Statistical significance was calculated by Fisher's exact test), (D) HLA-B*35:01 $core_{143-151}$ TVIEYLVSF (Statistical significance was calculated by Fisher's exact test), (E) HLA-B*18:01 $core_{36-44}$ KEFGASVEL (Statistical significance was calculated by Fisher's exact test) and (F) HLA-B*40:01 $core_{36-44}$ KEFGASVEL (Statistical significance was calculated by Fisher's exact test). Positions with p <0.05 by Fisher's exact test or posterior probability ≥0.8 in HAMdetector are colored in red. The results of the Fisher's exact test for all HAMs and the favored amino acid are shown. HAM, HLA-associated mutational state.

proteins. All posterior probabilities are shown for the different proteins in Fig. 3. When precore/core sequences were analyzed, posterior probabilities >0.8 were calculated for a total of 295 residues, consistent with strong evidence for HLA class I selection (Fig. 3B). In contrast, there were no posterior probabilities >0.8 when HLA class I genotypes were randomly assigned to the sequences (Fig. 3A), confirming that HLA class I genotypes strongly contribute to HAMdetector results. In the majority of cases with high posterior probabilities (212 of 295; 71.9%), the amino acids were variations from the majority consensus sequence. However, there were also instances (83 of 295) where high posterior probabilities were observed for the majority consensus amino acid. Similar to what has been observed in HIV and HCV, escape mutations may have accumulated at the population level and become the majority consensus residue.[23–29] Notably, evidence for selection of the consensus amino acid was more frequently observed for high-frequency HLA alleles in our cohort. For example, selection of consensus was linked to the high-frequency allele HLA-A*02 in 18.1% of cases, whereas selection of variations from consensus was driven by HLA-A*02 in only 6.6% of cases ($p = 0.0046$).

Interestingly, there were strong differences between the different HBV proteins. In contrast to the precore/core protein, only a few positions had posterior probabilities ≥0.8 in the viral envelope protein (large HBsAg; Fig. 3C) and the HBx protein (Fig. 3D), suggesting that HLA class I selection is less reproducible here. In HBV pol, posterior probabilities ≥0.8 were mainly observed in the N-terminal region of the protein that overlaps with the core region on the HBV genome (Fig. 3E). The complete list with all posterior probabilities for all HBV proteins are provided as described in the data availability statement.

Although HAMdetector integrates information on HLA class I-binding motifs into the model, it does not incorporate existing information on HLA class I-restricted epitopes in HBV. There are several possible mechanisms for increased evidence of HLA class I-associated selection pressure on the precore/core protein. These include a higher frequency of epitopes in the precore/core region or stronger selection pressure on epitopes in the precore/core region. To address this, all previously described and fully mapped HLA class I-restricted epitopes from the immune epitope database were analyzed in more detail. The complete list of epitopes with the respective posterior probabilities are available as described in the data availability statement. Although the number of described and experimentally matched epitopes is higher for the polymerase (54 epitopes) than the core protein (42 epitopes), the maximum posterior probabilities were higher for residues within the core epitope region (Fig. 3E). The previously described epitopes in HBsAg and HBx also showed lower maximum posterior probabilities compared to epitopes in the core protein. Taken together, the results are consistent with a higher degree of HLA class I-associated selection pressure on epitopes in the HBV precore/core protein compared to the remaining HBV proteins.

Viral adaptation to HLA class I-associated selection pressure correlates with markers of viral replication

The posterior probability of a given residue is a quantitative measure of the probability that the residue is a HAM. We next sought to establish a score as a quantitative measure for the level of adaptation to HLA class I expression in a complete protein. To calculate such an "*adaptation score*", we created a majority consensus reference sequence for each genotype and compared it to the individual sequences of the patient. The adaption score of an individual sequence was then calculated as the sum of all maximum posterior probabilities, for all relevant HLA class I alleles of the individual patient, in positions that differed from the consensus sequence (Fig. 4A). Accordingly, viral proteins with high sequence homology to the consensus sequence tend to have low adaptation scores. Conversely, viral proteins with multiple differences from the consensus sequence tend to have a high adaptation score if the differences are likely to be HAMs according to the HAMdetector results.

We then investigated whether these adaptation scores correlated with markers of viral replication. An important marker for the clinical classification of chronic HBV infection is HBeAg serostatus.[2] Therefore, we compared the HLA class I adaption scores between 58 HBeAg-positive patients and the 350 HBeAg-negative patients for whom the HBeAg serostatus was available. Consistent with a higher degree of CD8 T-cell selection pressure in HBeAg-negative hepatitis B, HLA class I adaptation scores were significantly higher in HBeAg-negative patients compared to HBeAg-positive patients (Fig. 4B; $p < 0.0001$). There was a negative correlation between the HLA class I adaptation scores and the serum HBV DNA concentrations (Fig. 4C; $p < 0.0001$; $r = -0.2993$) and the HBsAg level (Fig. 4D; $p < 0.0001$; $r = -0.2867$). Notably, the adaptation scores also positively correlated with age (Fig. 4E; $p < 0.0001$; $r = 0.3336$), suggesting that adaptation to HLA class I-associated selection pressure increases over time.

Taken together, our analysis of all HBV proteins suggested different levels of HLA class I-associated selection pressure, with particularly strong selection pressure on the HBV precore/core protein. Furthermore, quantification of the levels of HLA class I adaptation for individual isolates revealed differences, which correlate with markers of replication and age. HBeAg-positive HBV infection is associated with low levels of HLA class I adaptation. Moreover, high levels of HLA class I adaptation were observed in patients with low viral load and low HBsAg levels.

Discussion

Selection of escape mutations in targeted epitopes of the CD8 T-cell response has been well described in chronic viral hepatitis.[9,30] In HBV infection, there is also strong evidence for selection of escape mutations in the epitope core$_{18\text{-}27}$, which is supported by functional experiments showing the impact of epitope variants on the CD8 T-cell response.[10,11,13,17,31] Reproducible immune selection of virus mutations in hosts sharing the same HLA class I allele can be detected as statistical associations between viral sequence polymorphisms and HLA class I alleles at the population level.[13,32–34] In a previous analysis of viral sequences from the HBV precore/core we found strong evidence for HLA class I-associated selection pressure and were able to use a viral sequencing approach for identification of novel CD8 T-cell epitopes.[13] Here, we extended the analysis to all HBV open-reading frames, including the proteins precore/core, pol, HBsAg and HBx. In this analysis we utilized HAMdetector, which was specifically

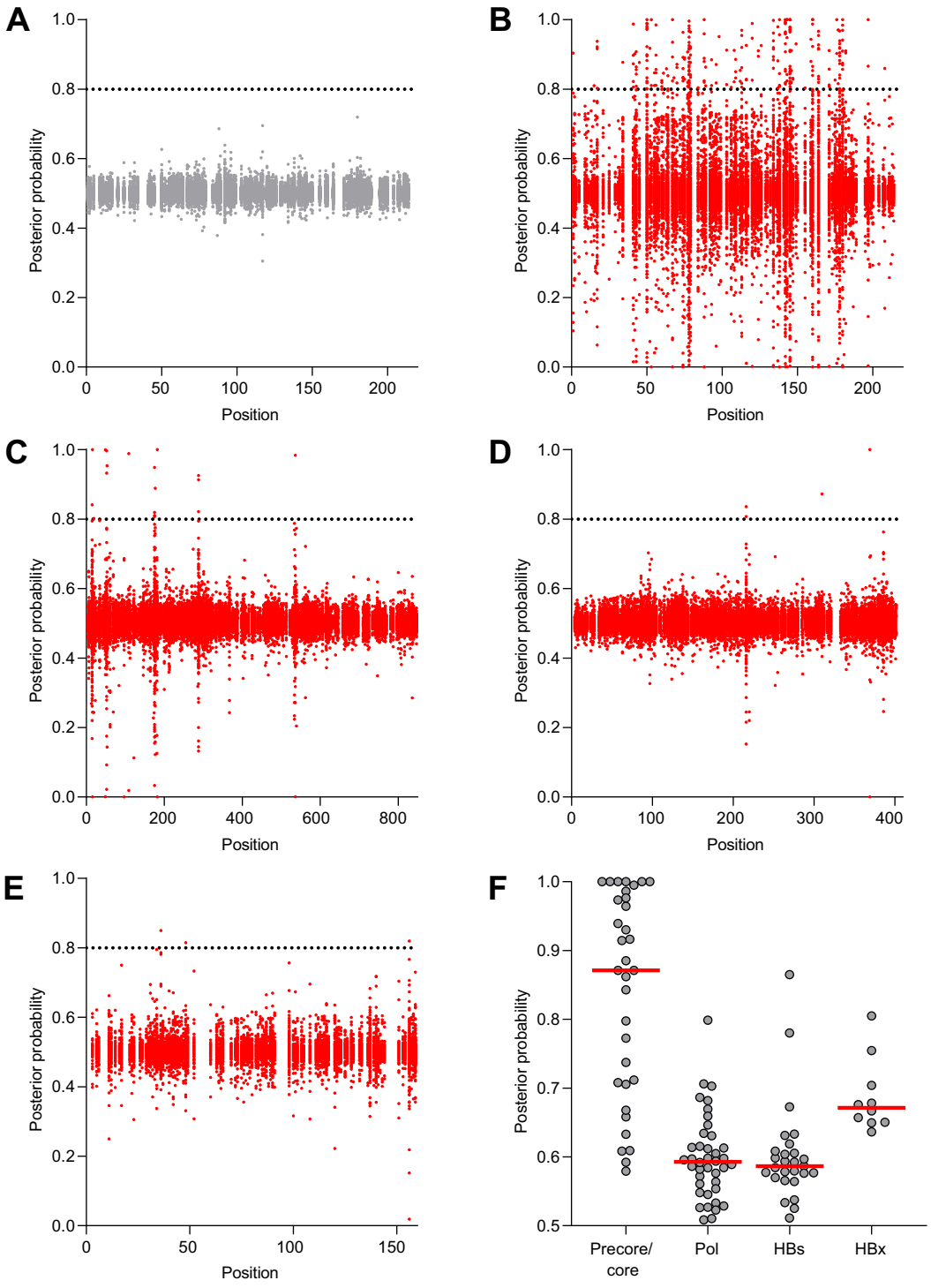

Fig. 3. HLA-associated mutational states in HBV proteins. Alignments of the four proteins precore/core, pol, HBsAg and HBx were analyzed with HAMdetector. (A) HAMdetector results for the precore/core protein with randomly assigned HLA class I genotypes (Posterior probability was calculated using the Bayesian approach, as implemented in the HAMdetector). (B) HAMdetector results for the precore/core protein with correctly assigned HLA class I genotypes (Posterior probability was calculated using the Bayesian approach, as implemented in the HAMdetector). (C-E) HAMdetector results for (C) pol, (D) HBsAg and (E) HBx (Posterior probability was calculated using the Bayesian approach, as implemented in the HAMdetector). The dotted line represents a 0.8 posterior probability threshold. In a previous study[22] posterior probabilities ≥0.8 were strongly indicative of true CD8 T-cell epitopes. (F) Previously described and fully mapped HLA class I-restricted epitopes were retrieved from a public database (IEDB). For each epitope, the maximal posterior probability for all amino acid position of the epitope was calculated for the relevant restricting HLA class I type and is shown for epitopes located in precore/core, pol, HBsAg and HBx (Posterior probability was calculated using the Bayesian approach, as implemented in the HAMdetector). HAM, HLA-associated mutational state; HBsAg, hepatitis B surface antigen; HBx, hepatitis B x protein.

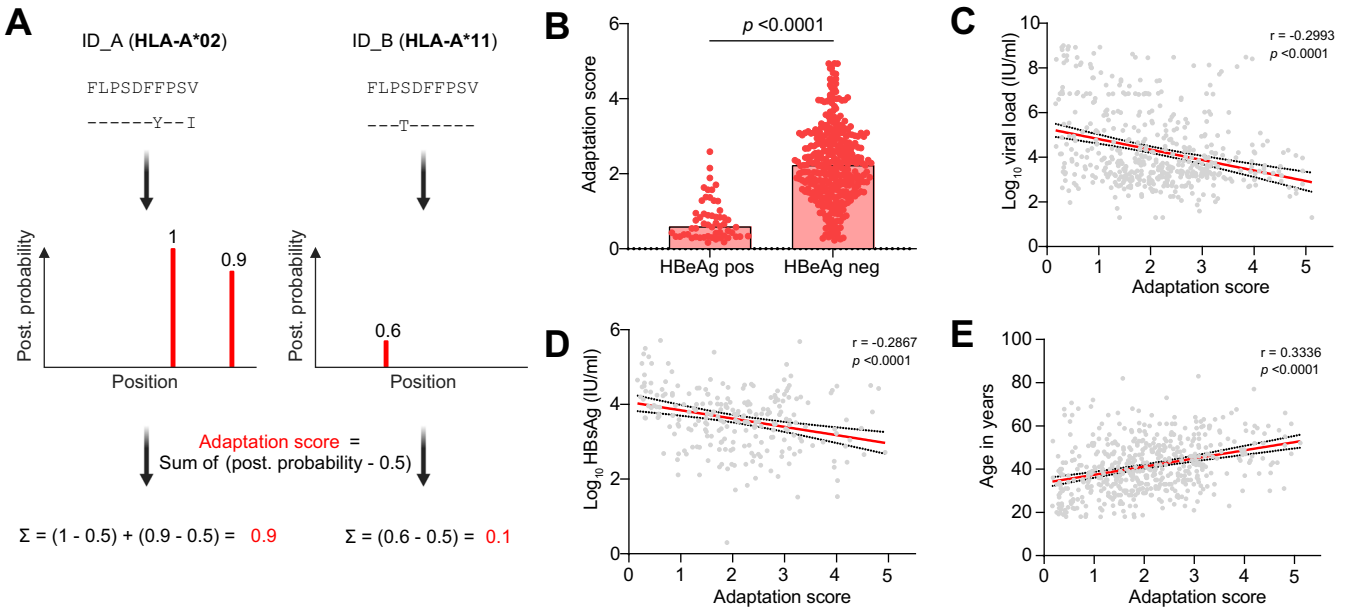

Fig. 4. Adaptation of HBV to HLA class I-associated selection pressure correlates with markers of HBV replication. (A) Schematic illustration of the adaptation score calculation. The adaptation score represents the number of amino acid substitutions in each sequence compared to a consensus sequence and uses maximum posterior probabilities from HAMdetector to weigh the substitutions based on the likelihood that they were selected by HLA. (B) Comparison of adaptation scores for isolates from HBeAg-positive and HBeAg-negative HBV infection as determined by the HBeAg serostatus (Statistical significance was calculated by Welch's t-test). (C) Correlation of adaptation scores of viral sequences with HBV DNA concentrations in serum (Statistical significance was calculated by Simple linear regression). (D) Correlation of adaptation scores of viral sequences with HBsAg levels in serum (Statistical significance was calculated by Simple linear regression). (E) Correlation of adaptation scores with age (Statistical significance was calculated by Simple linear regression). The red line represents the linear regression analysis and the dotted line the margins of the 95% confidence intervals. HAM, HLA-associated mutational state; HBeAg, hepatitis B e antigen.

designed for detection of HAMs in viral genomes.[22] Compared to conventional statistical approaches utilizing HLA class I genotypes and mutation frequencies in the viral genome, HAMdetector has the advantage that additional information such as HLA class I motifs and the phylogeny of the viral sequences are integrated into one model. This allows for more accurate detection of HAMs by increasing the sensitivity and decreasing the rate of false positive results.[22] HAMdetector has been applied to different datasets of viral sequences including HIV, HBV and HDV.[18,22] The in-depth analysis of residues under selection pressure provided here may promote future studies on epitope identification and mapping that will be required when exploring the functional differences of CD8 T cells directed against different HBV proteins.

In most cases, the consensus amino acid will represent the viral state in the absence of immune pressure. However, this does not hold true for all situations when viral infections are studied at the population level. There are multiple instances where the consensus sequence of circulating viral isolates within a population is modulated by immune selection. For example, in HIV and HCV, the accumulation of escape mutations at the population level and their fixation in the consensus sequence has been well-documented.[23–29] Several mechanisms contribute to this accumulation, including the transmission of escape mutations to new hosts,[35,36] lack of reversion in the absence of immune pressure when the fitness cost of the escape variant is low,[28,35,37] and selection by highly frequent HLA alleles in the population.[24,27,38] Together, these mechanisms influence substitution frequencies in circulating isolates, potentially leading to the replacement of the original consensus residue with an escape residue. Indeed, evidence from HIV and HCV suggests that consensus sequences may differ between populations due to variations in HLA allele frequencies.[24,27,38] This is consistent with our dataset, where 28.1% of residues with evidence for immune selection pressure correspond to the majority consensus amino acid. This suggests that HBV may already have adapted to some extent to high-frequency HLA alleles at the population level.

When HAMdetector was applied to alignments of the different HBV proteins, the results suggested that the frequency of HAMs was substantially higher in the precore/core protein compared to the other proteins. Importantly, systematic studies of the breadth of the CD8 T-cell response against different HBV proteins are lacking. It is therefore unclear if the overall epitope density in the core protein is higher or if the individual CD8 T-cell responses against epitopes in core exert more reproducible selection pressure. Although not definitive, our analysis of described CD8 T-cell epitopes in HBV proteins from a public database suggests that reproducible selection of escape mutations seems to be a characteristic of core epitopes. This would suggest either host differences in the quantity or quality of the CD8 T-cell response between viral proteins or differences between the targeted proteins in their ability to accommodate escape mutations. In line with differences between the magnitude of the CD8 T-cell response against different HBV proteins, more robust CD8 T-cell responses against the core protein than the envelope protein have been reported in chronic HBeAg-negative infection, but this difference was not fully consistent and depended on the disease stage.[39–41] Interestingly, the phenotype of CD8 T cells directed against the core protein also differed from that of CD8 T cells directed against HBV pol,[42,43] suggesting that functional

differences may contribute to the higher degree of selection pressure on the core protein. That said, we cannot exclude that the ability of the virus to accommodate escape mutations differs between viral proteins. Of note, as an essential part of the virus particle, the core protein is subject to functional constraints that limit its sequence diversity. When we compared the entropy at the amino acid level between the HBV proteins, we did not find any differences for amino acid positions with strong support for selection in HAMdetector (Fig. S2).

Importantly, based on the secreted form of the precore protein, different stages of chronic HBV infection are distinguished.[2] During HBeAg-positive HBV infection, the precore protein is translated and further processed and secreted as the HBeAg, which can be detected in serological assays. The exact functional role of the secreted HBeAg is not fully understood, but it has been associated with a state of "tolerance" characterized by high HBV DNA concentrations and no or only mild inflammation in the liver.[3] In our analysis, HBeAg-positive HBV infection was associated with lower levels of HLA class I adaptation compared to HBeAg-negative HBV infection. This is in line with prior studies of the precore/core protein where substitutions were enriched during the HBeAg-negative phase of infection.[13,17,44–46] Collectively, the data strongly support that the sequence diversity in HBV core is strongly influenced by CD8 T-cell pressure in HBeAg-negative stages but that there is less selection pressure in HBeAg-positive infection.

Different levels of adaptation to HLA class I-associated selection pressure also correlated with HBV DNA and HBsAg concentrations in serum. High levels of adaptation to HLA class I-associated selection pressure were observed in isolates from patients with low viral load and low HBsAg levels. The correlations also suggest that viral adaptation to HLA class I pressure may cause fitness costs by impairing viral replication. Notably, the negative correlation was mainly driven by HLA adaptation of the precore/core protein, for which impaired virion production was previously observed in HBeAg-negative hepatitis.[47] Analysis of the mechanisms by which substitutions in the precore/core protein may cause impairment of replication would require further studies.[48] Importantly, it is also possible that viral fitness is not impaired and low HBV DNA concentrations are the consequence of the immune response. In this case, high HLA class I adaptation scores may simply be a marker of a functional CD8 T-cell response that is responsible for inhibition of viral replication.

The cohort studied here came from different institutions in Central Europe and represents the patients in the hepatology outpatient clinics from this area, with a predominance of genotypes D and A. Accordingly, although the analysis tool controls for the underlying phylogeny of the sequences, there may be genotype-specific differences that were not detected in this study. The viral sequence was obtained prior to antiviral treatment; however, this may lead to a selection bias in our cohort when patients with low viral load and normal transaminase levels were preferentially included. We specifically tried to include HBeAg-positive patients; however, untreated HBeAg-positive HBV infection is uncommon in such cohorts and was present in only 14.2% of our cohort. Nevertheless, the strong difference in the level of HLA class I adaptation between HBeAg-positive and HBeAg-negative patients is striking. Unfortunately, we were only able to perform cross-sectional studies between these groups. Thus, it is unclear if HAMs are only selected upon transition from HBeAg-positive to HBeAg-negative HBV infection or if this is a continuous process during persistent HBeAg-negative HBV infection. Selection of variants and increased substitution rates in the HBV quasispecies have been described during the HBeAg seroconversion phase.[44] However, the level of adaptation also correlated with age. Using patient age as a proxy for duration of infection, this strongly suggests that viral genomes continuously accumulate HAMs over time. More detailed longitudinal analyses of viral sequences combined with CD8 T-cell studies would be required to address this, which is difficult to perform as most patients with relevant HBV replication receive antiviral treatment to prevent potential liver disease.

In summary, our study provides important insights into the extent of HLA class I-associated selection pressure on HBV and highlights that CD8 T-cell pressure strongly contributes to sequence diversity in the HBV core protein. Moreover, different levels of adaptation to CD8 T-cell pressure were observed, which correlate with markers of viral replication. These different levels of adaptation are relevant for the further development of T cell-based therapeutic strategies, as they may represent different levels of susceptibility to immune therapy. We would hypothesize that less adapted isolates would be more susceptible to T-cell therapy, which needs to be further studied.

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Abbreviations

HAM, HLA-associated mutational state; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen; HBx, hepatitis B x protein; PBMC, peripheral blood mononuclear cells; TCR, T-cell receptor.

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Authors' contributions

The project was conceived by TS, AW and JT. Experiments were performed by TS, JP, MD, CM, EA, JLM, MM, HK, AK, SG, FH and AW. EA, JSW, GL, HK, MC, AK, SG, HHB, PAH, MKM, RT, HW, JN, FMH, TL and CNH contributed samples

and clinical data. All authors contributed to the interpretation of the results. The original draft was written by AW and JT and finalized with input from all authors. All authors contributed to the article and approved the submitted version.

Conflict of interest

TS, JP, MD, SG, DH, FMH, JLM, TL, CM, AK, ESA, CNH, DH, MM, RT, PH declare no conflict of interest. JT reports honoraria for lectures from AbbVie and GSK. AW has received grants for HCV genomic surveillance from Gilead Sciences. HB reports honoraria for lectures or presentations from AbbVie and Gilead Sciences, travel support from AbbVie and Gilead Sciences, and participates on advisory boards for AbbVie, Gilead Sciences, and Ipsen. MKM reports funding from Gilead Sciences for HBV immunology research and received consulting fees from Astrivax and Moderna. MC reports consulting fees from Gilead Sciences, AbbVie, MSD Sharp & Dohme, Falk Foundation, and is the medical CEO of the German Liver Foundation. JN reports grants from the German Research Council, the German Center for Infection Research, the Hector Foundation, and the German Cancer Aid. JSzW reports grants from EU Horizon 20/20, DZIF, DFG CRC1328. HW reports grants from Abbott, Biotest, consulting fees from Abbott, Bristol-Myers-Squibb, F. Hoffmann-La Roche Ltd., Gilead Science, GlaxoSmithKline, Janssen, Roche Diagnostics International Ltd., Vir Biotechnology Inc. and honoraria from Biotest Ag and Gilead Science. HK reports grants from the German Center for Infection Research, EU Horizon Health, German Research Foundation, and internal Funds from Hannover Medical School and support for attending the International Delta-Cure Meeting and EASL/AASLD Masterclass. GL reports funding from NIH.

Please refer to the accompanying ICMJE disclosure forms for further details.

Data availability statement

The authors openly provide the data presented in this manuscript through the OSF Home service (https://osf.io/e7spw/?view_only=0e72873cc5fa413a9a3c8b0d2b9c9e37). Sequence data are available at GenBank (MZ043025-MZ043097; MZ097624-MZ097884).

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Supplementary data

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