Pilot Study Using Machine Learning to Identify Immune Profiles for the Prediction of Early Virological Relapse After Stopping Nucleos(t)ide Analogues in HBeAg-Negative CHB

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Treatment with nucleos(t)ide analogues (NAs) may be stopped after 1-3 years of hepatitis B virus DNA suppression in hepatitis B e antigen (HBeAg)-negative patients according to Asian Pacific Association for the Study of Liver and European Association for the Study of Liver guidelines. However, virological relapse (VR) occurs in most patients. We aimed to analyze soluble immune markers (SIMs) and use machine learning to identify SIM combinations as predictor for early VR after NA discontinuation. A validation cohort was used to verify the predictive power of the SIM combination. In a *post hoc* analysis of a prospective, multicenter therapeutic vaccination trial (ABX-203, NCT02249988), hepatitis B surface antigen, hepatitis B core antigen, and 47 SIMs were repeatedly determined before NA was stopped. Forty-three HBeAg-negative patients were included. To detect the highest predictive constellation of host and viral markers, a supervised machine learning approach was used. Data were validated in a different cohort of 49 patients treated with entecavir. VR (hepatitis B virus DNA ≥ 2,000 IU/mL) occurred in 27 patients. The predictive value for VR of single SIMs at the time of NA stop was best for interleukin (IL)-2, IL-17, and regulated on activation, normal T cell expressed and secreted (RANTES/CCL5) with a maximum area under the curve of 0.65. Hepatitis B core antigen had a higher predictive power than hepatitis B surface antigen but lower than the SIMs. A supervised machine-learning algorithm allowed a remarkable improvement of early relapse prediction in patients treated with entecavir. The combination of IL-2, monokine induced by interferon γ (MIG)/chemokine (C-C motif) ligand 9 (CCL9), RANTES/CCL5, stem cell factor (SCF), and TNF-related apoptosis-inducing ligand (TRAIL) was reliable in predicting VR (0.89; 95% confidence interval: 0.5-1.0) and showed viable results in the validation cohort (0.63; 0.1-0.99). Host immune markers such as SIMs appear to be underestimated in guiding treatment cessation in HBeAg-negative patients. Machine learning can help find predictive SIM patterns that allow a precise identification of patients particularly suitable for NA cessation. (*Hepatology Communications* 2021;5:97-111).

Chronic hepatitis B is a major challenging health problem, with 250 million chronically infected patients worldwide.[1] Treatment options are either pegylated interferon alfa (PEG-IFN) or nucleoside or nucleotide analogues (NAs).[2] PEG-IFN has the advantage of finite treatment duration, but it has to be administered subcutaneously and side effects restrict its use.[2] Oral NAs are safe and highly effective in terms of hepatitis B virus (HBV) DNA suppression, but treatment duration is not finite.

In hepatitis B e antigen (HBeAg)–negative patients, usually treatment should only be stopped after hepatitis B surface antigen (HBsAg) clearance, which is a rare event in this setting.[3,4] Based on modeling studies, HBeAg-negative patients would need to be treated for a median duration of 39-52 years until HBsAg loss.[5,6] However, based on experience primarily from Asia, some guidelines recommend that NA therapy can be stopped in patients without cirrhosis after 1-3 years of successful therapy.[2,7] Nevertheless, HBV relapse is still frequent, and only 30% remain in virological remission in the long-term follow-up after cessation of therapy.[8] Some patients may even experience severe alanine aminotransferase (ALT) flares associated with increased risk for liver decompensation.[8,9] Thus, frequent monitoring after cessation of NA therapy is required.[2] So far, there are no valid biomarkers to predict the outcome after stopping NA treatment in HBeAg-negative patients. Several studies have shown that low HBsAg level at the time before stopping NA therapy is associated with virological remission subsequently having less clinical relapse[10-13] but the predictive value is still unsatisfactory. Other virologic markers that may help

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to predict virological relapse (VR) are HBV RNA or hepatitis B core antigen (HBcAg), which may be better surrogates of transcriptional covalently closed circular DNA.[14,15] A recent study has shown that detectable HBV RNA and HBcAg were associated with severe ALT flares after NA withdrawal.[16]

So far, no host marker has been considered as a biomarker to predict the outcome after stopping NA therapy. Multi-omics technologies have advanced and become affordable.[17] Because virological remission after stopping NA therapy is most likely controlled by host immune responses, host immune marker measured in the plasma or serum might be a promising biomarker in this setting. However, it is unlikely that just one parameter will serve as a predictive biomarker, so a multiomics approach might be needed to identify a valuable marker panel. Artificial intelligence technologies such as machine learning (ML) may be helpful in identifying the best combination of markers,[18] and were used here. In addition, a validation cohort was implemented to validate the results in an independent cohort.

Thus, the aim of our study was to analyze 47 soluble immune markers (SIMs) at three different time points during the NA treatment, and use ML to identify SIM combinations as predictors for early VR, defined as HBV DNA ≥ 2,000 IU/mL until week 24 after NA discontinuation in HBeAg-negative patients, and confirm these results in the validation cohort. We also compared the performance of SIMs with the viral markers HBsAg and HBcAg.

Materials and Methods

COHORT

The derivation cohort was selected from the ABX 203-002 study. The ABX 203-002 trial was a phase 2b-3, open-label, randomized, comparative study that assessed the efficacy of the ABX 203 vaccine to maintain control of HBV infection after cessation of antiviral treatment with NAs in adult HBeAg-negative patients with chronic hepatitis B in the Asia Pacific region. The design, inclusion, and exclusion criteria were as described previously.[19] For the present analysis, we included only patients who were HBeAg-negative before the start of NA treatment. Subjects in the study also had to have HBV DNA < 40 IU/mL as well as ALT and aspartate transaminase levels less

than or equal to the upper limit of normal each, at least 1 year before screening. As additional serum samples were needed for the measurement of SIMs, only patients who participated in the additional serum sampling with sufficient clinical data and serum samples at all three time points during therapy were included (weeks 0, 12, and 24).

To eliminate possible confounders for SIM measurement, we excluded patients who received the investigational vaccine. A detailed description is shown in Fig. 1A.

The validation cohort was derived from the Chang Gung Memorial Hospital (CGMH) off-Nuc cohort.[20] Briefly, mono-infected, HBeAg-negative patients had stopped NA therapy after demonstration of undetectable HBV DNA on three occasions, each more than 6 months apart according to Asian Pacific Association for the Study of Liver (APASL) guidelines.[20,21] After end of therapy, patients were monitored every 1-1.5 months in the first 3 months, then every 3 months, along with HBV-DNA assay for a total of 1 year and every 3-6 months thereafter (more frequently in cases of virological or clinical relapse).[20]

The procedures of this study were in accordance with the Declaration of Helsinki and approved by the ethics committee (ClinicalTrials.gov registration No. NCT02249988).[20] All patients gave written, informed consent to participate in this study.[20]

STRUCTURE OF THE STUDY AND DEFINITIONS

All patients received at least 2 years of NA treatment before enrollment into the study. Subjects then received NA therapy for another 24 weeks after screening, and then stopped treatment (Fig. 1B). Patients were followed up every 2 weeks for the first 8 weeks after cessation of treatment, and from there on every 4 weeks. The primary endpoint of the study was the percentage of subjects with HBV DNA < 40 IU/mL at week 24 after NA discontinuation. Serum samples were taken during therapy at weeks –24, –12, and 0 (end of therapy), after therapy was stopped (weeks 12 and 24), and at the end of the study. The end-of-study time point was variable: For subjects who relapsed, it was defined as an increase in HBV DNA > 2,000 IU/mL [European Association for the Study of Liver (EASL), American Association for the Study of Liver Diseases (AASLD), APASL]); for those who did not relapse, it
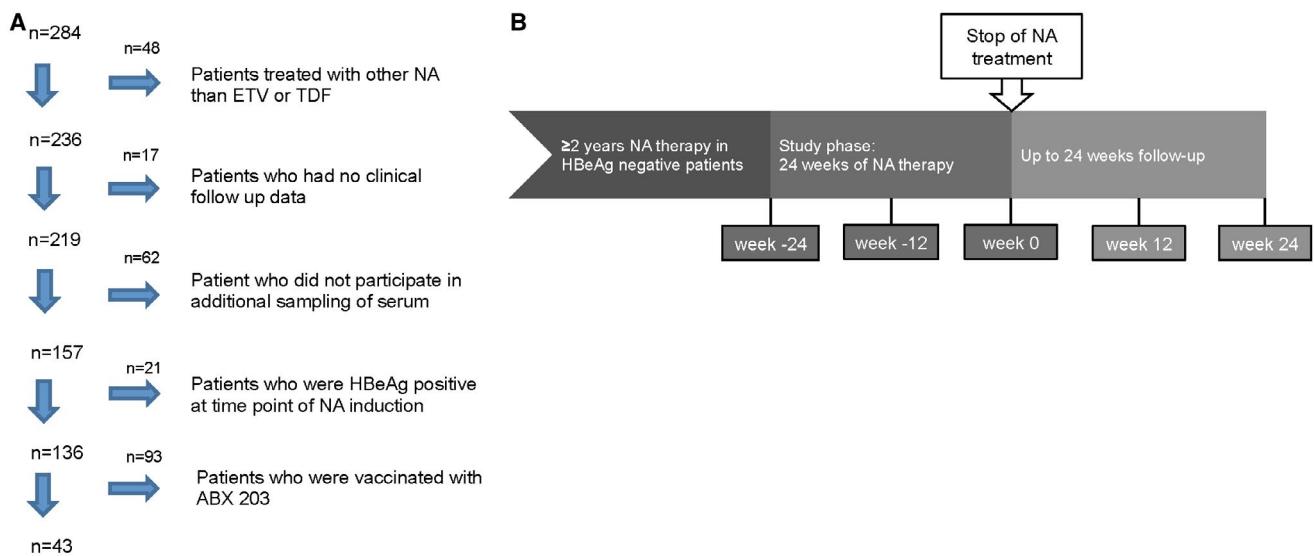
FIG. 1. (A) Selection of the study population. Patients were included from the ABX 203-002 study. The ABX 203-002 trial was a phase 2b-3, open-label, randomized, comparative study to assess the efficacy of the ABX 203 vaccine to maintain control of hepatitis B disease after cessation of antiviral treatment with NA in adult HBeAg-negative patients with chronic hepatitis B in the Asia Pacific region. (B) Study structure: All patients received at least 2 years of NA treatment before enrollment into the study. In the study phase, patients received another 24 weeks of NA treatment and then discontinued treatment at week 0. Blood samples were drawn at weeks –24, –12, and week 0. Patients were followed for 24 weeks. The primary endpoint was percentage of subjects with HBV DNA < 40 IU/mL at week 24 after NA discontinuation.

was defined as the visit at week 24 after stopping the NA therapy.

LABORATORY TESTING

Clinical laboratory tests were performed according to the Good Clinical Laboratory Practice standards. In the derivation and validation cohorts, the Roche Cobas AmpliPrep/Cobas TaqMan HBV Test (version 2.0; Roche Diagnostics, Basel, Switzerland) with a lower limit of < 20 IU/mL was used for HBV-DNA quantification. Quantitative levels of HBcAg were determined using the Lumipulse G HBcAg assay (Fujirebio Europe, Belgium; Fujirebio Japan). Samples were handled according to the manufacturer's instructions. The assay's validated measurement range is from 3 log to 7 log U/mL. However, if HBcAg levels are lower than 3 log U/mL, the machine indicates it down to 2 log U/mL in HBcAg-positive samples.[22] In the cases of undetectable HBcAg levels (below 2 log U/mL), it is not possible to distinguish between HBcAg-negative samples and samples that are detectable but not quantifiable.[23] If HBcAg levels were below 2 log U/mL, they were calculated as 2 log U/mL

for statistical analysis.[23] For measuring of SIMs, we used Bio-Plex Pro Human Cytokine, Chemokine, and Growth Factor Assay (Bio-Rad Laboratories, Hercules, CA), which includes the following 48 SIMs: CTACK/CCL27 (cutaneous T-cell-attracting chemokine), eotaxin/CCL11 (eosinophil chemotactic protein), FGF-β (basic fibroblast growth factor), G-CSF (granulocyte colony-stimulating factor), GM-CSF (granulocyte-macrophage colony-stimulating factor), GRO-α/CXCL1 (growth-regulated protein alpha), HGF/scatter factor (hepatocyte growth factor), IFN (interferon)-α2, IFN-μ, interleukin (IL)-1α, IL-1β, IL-1ra (receptor antagonist), IL-2, IL-2Rα (receptor alpha chain), IL-3, IL-4, IL-5, IL-6, IL-7, IL-8/CXCL8, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-16, IL-17A, IL-18, IP-10/CXCL10 (interferon-γ-inducible protein 10), LIF (leukemia inhibitory factor), MCP (monocyte chemotactic protein)-1/CCL2, MCP-3/CCL7, M-CSF (macrophage colony-stimulating factor), MIF (macrophage migration inhibitory factor), MIG/CXCL9 (monokine induced by interferon γ), MIP (macrophage inflammatory protein)-1α/CCL3, MIP-1β/CCL4, PDGF-BB (platelet-derived growth factor BB), RANTES/CCL5

(regulated on activation, normal T cell expressed and secreted), SCF (stem cell factor), SCGF-β (stem cell growth factor-beta), SDF-1α (stromal cell-derived factor 1), TNF (tumor necrosis factor)-α, TNF-β/lymphotoxinα, TRAIL (TNF-related apoptosis-inducing ligand), VEGF (vascular endothelial growth factor), and β-NGF (beta–nerve growth factor) (Supporting Table S1). Measurement was performed according to manufacturer's instructions. To prevent interassay variation, all samples of each cohort were measured in one run. The derivation and validation cohort were measured at different time points and laboratories. However, they used the same immunoassays and laboratory equipment. Detectable SIMs at the lower limit of quantification were used according to the method implemented by Beal: The lowest measurable value of the particular analyte given by the instruction manual was divided by two.[24] VEGF had to be excluded from further analysis due to agglutination error in the validation cohort.

STATISTICAL ANALYSIS

Correlations Among Cytokines, Viral Markers, and Early Relapse

Pearson correlation coefficients were obtained with the function pearsonr() in the scipy.stats module (https://docs.scipy.org/doc/scipy/reference/stats.html) and then used to assess correlations among cytokines, viral markers (HBsAg and HBcAg), and the target variable of early VR and nonrelapse at each time point.

Machine Learning Approach

A supervised ML approach for classification on labeled data of early VR versus nonrelapse after therapy cessation was implemented using the scikit-learn 0.21.2 package.[25] A cohort of 43 HBeAg-negative patients with chronic hepatitis B (27 early relapsers and 16 nonrelapsers) and measurements of 47 SIMs and two viral markers (HBsAg and HBcAg) at different time points before therapy cessation (weeks –24, –12, and 0) were included. Input feature data (i.e., SIMs and viral markers) for ML algorithms were standardized by removing their mean and scaling to unit variances while using pipelines of the sklearn tool to avoid data leakage during cross-validation. Early

VR was used as the binary target variable for the supervised ML classifiers.

Feature selection was performed to identify the most predictive subset of features (i.e., SIMs). This process consisted of the following main steps: (1) Find different subsets of k-best features (with $k = 15$) at week 0 as outputs of different feature selection approaches; (2) select the most predictive feature combinations from each feature subset in step 1; and (3) select from all resulting feature subsets in step 2 the most predictive (on average) when individually evaluated on the three time points (weeks –24, –12, and 0).

In the first step, three independent alternative categories of algorithms[26] were implemented:

- *Filter methods* assign a score to each feature and allow to select the k-best feature subset based on a given metric. Herein, we scored features by their (i) variance, (ii) Pearson correlation coefficient with the target variable, (iii) chi-squared tests, and (iv) the analysis of variance F-tests. The k top-ranked features sorted in descending order according to their scores were obtained for each metric. These methods were implemented using the SelectKBest function in sklearn, which removes all but the k-highest scoring features for each metric.
- The *Wrapper method* considers feature selection as a search problem, in which a predictive model is used to rank features by recursive feature elimination and cross-validation using the RFECV function in sklearn. This assigns a score to each feature based on model accuracy. The implemented wrapper method used logistic regression (LGR), C-support vector classifier (SVC), and the SGDClassifier as predictive models, which implement logistic regression with elastic net penalty on the loss function. The k top-ranked features were obtained for each predictive model.
- *Embedded methods* perform feature selection during the process of training predictive models. We implemented this approach through the SelectFromModel function in sklearn, which is a meta-transformer for selecting features based on importance weights. LGR, SVC, and SGDClassifier were considered predictive models for SelectFromModel. Features were ranked based on their importance weights, and the k-top features with the highest weights were obtained for each model.

All feature combinations verified in both the wrapper and embedded approaches were equally

evaluated using the default parameter sets of predictive models (i.e., without hyperparameter optimization).

In the second step, additional wrapper methods were applied to each set of k top-ranked features from the filter, wrapper, and embedded methods in step 1. We implemented forward and backward stepwise feature-selection approaches[27] coupled with the classification models LGR, SVC, and SGDClassifier with their default parameter sets to identify smaller feature subsets of more predictive value.

In step 3, all resulting feature subsets in step 2 were then individually evaluated on each time point and for each model with cross-validation and hyperparameter optimization. The feature subset with most predictive value (on average) of the three time points (i.e., weeks 0, –12, and –24) was selected.

To evaluate the quality of the identified feature subset, hyperparameter optimization with a 5-fold cross-validation repeated 20 times was individually conducted on different classification models. Among the five classifiers (LGR, SVC, SGDClassifier, k-Nearest Neighbors, and Random Forest[25,27]), SVC showed the best cross-validation accuracy at all time points and was selected as the reference predictive model. Hyperparameters of the SVC model were optimized on each individual time point with cross-validation, and the resulting accuracy was reported. In this study, we always considered 5-fold cross-validation repeated 20 times, and hyperparameter optimization was performed using the GridSearchCV function in sklearn, which is the most widely used exhaustive grid search algorithm with cross-validation.[25] To report the predictive performance of the classification procedure, receiver operating characteristic (ROC) curves that plot the trade-offs between sensitivity and specificity were computed by means of the function roc_curve() of the sklearn.metrics module. The mean area under the curve (AUC), SDs, and 95% confidence intervals (CIs) resulting from cross-validation were reported.

Results

CLINICAL PARAMETERS OF THE DERIVATION COHORT

All patients were HBeAg-negative before the start of NA therapy. Twenty-eight patients were treated with entecavir (ETV; 65%) and 15 patients with tenofovir (TDF; 35%) (Table 1). Of the 43 patients, 27 experienced early VR (HBV DNA > 2,000 IU/mL) until week 24 after stopping therapy, whereas 16 patients had no relapse (Table 1). The median relapse time point was 20 weeks after cessation of NA treatment. No significant differences in terms of clinical and virological parameters at baseline (week 0) were noticed between patients with and without early relapse, except for type of antiviral therapy (P = 0.023) (Table 1). The two viral markers HBsAg and HBcAg were not significantly different between the two groups when NA treatment was stopped (Table 1).

SINGLE SIMs AND PREDICTION OF EARLY VR

Forty-seven different SIMs were analyzed. Figure 2 shows a heat map with the different SIM expression levels of all single patients (relapsers and nonrelapsers) in comparison to the median of the entire cohort. For some SIMs, relapsers appear to show higher expression levels, whereas for others, the nonrelapsers have higher values (Fig. 2). To get the exact value of SIM that was higher in one of the two groups, we calculated the ratio of the medians between patients with and without early relapse (Fig. 3A). Of the 47 SIMs included in the analysis, 20 yielded higher medians in the nonrelapse group at week 0, 10 yielded higher medians in the relapse group, and the remaining 17 showed equal medians between the two groups (Fig. 3A). In the next step, we assessed whether SIM levels could predict VR. The SIMs that were most significantly linked with early VR at the time point of therapy withdrawal were IL-2, IL-6, MIP-1α/CCL3, RANTES/CCL5, and IL-7 (P values: 0.002, 0.021, 0.027, 0.039, and 0.042, respectively), with higher values in the relapse group for RANTES/CCL5 and lower values for IL-2, IL-6, IL-7, and MIP-1α/CCL3. IL-1α, IL-1β, MIG/CXCL9, IP-10/CXCL10, and IL-17A showed association with early VR to a lower degree (P values: 0.063, 0.063, 0.063, 0.069, and 0.088, respectively). The results for all cytokines are given in Supporting Table S2A-C for weeks 0, –12, and –24.

Next, we examined the predictive values for single SIMs and viral markers using ROC analysis. All markers showed an AUC ≤ 0.67 at any time point (Fig. 3B). The highest AUC values at NA

TABLE 1. BASELINE CHARACTERISTICS OF UNVACCINATED HBeAg-NEGATIVE PATIENTS (n = 43) AT THE TIME POINT OF NA CESSATION (WEEK 0)

Parameters Week 0	All Patients	Relapsers (Until Week 24)	Nonrelapsers (Until Week 24)	P Value (Relapsers vs. Nonrelapsers)
No. of patients	43	27	16	
Age (years)	53 (20-65)	51 (26-63)	56 (20-65)	0.371
Sex				1
Female	14 (33%)	9 (64%)	5 (36%)	
Male	29 (67%)	18 (62%)	11 (38%)	
Antiviral therapy				0.023
Entecavir	28 (65%)	14 (50%)	14 (50%)	
Tenofovir	15 (35%)	13 (87%)	2 (13%)	
Albumin (g/dL)	46 (38-50)	47 (42-50)	46 (38-50)	0.215
ALT (U/L)	20 (12-44)	20 (12-44)	23 (13-38)	0.119
Bilirubin (mg/dL)	8 (0-31)	8 (0-21)	9 (3-31)	0.679
HBcAg log (U/mL)	3.0 (2.0-5,4)	3.1 (2.0-5.4)	2.9 (2.0-4.4)	0.179
HBsAg (U/L)	987 (4-19,382)	847 (4-19,382)	1,125 (6-3,637)	0.839
Hemoglobin (g/L)	148 (111-167)	148 (111-164)	151 (114-167)	0.166
Platelets (/nL)	197 (105-385)	187 (117-317)	211 (105-385)	0.111

Note: Continuous data are presented as the median plus range. VR is defined as HBV DNA increase ≥ 2,000 IU/mL within 24 weeks after NA cessations.

stop (week 0) were achieved by IL-2, RANTES/CCL5, IL-17A, IP-10/CXCL10, and IL-6 (mean AUC, 95% CI: 0.65, 0.01-0.99; 0.65, 0.12-0.93; 0.63, 0.07-0.99; 0.60, 0.14-0.99; and 0.59, 0.5-0.83; respectively).

The viral markers HBsAg and HBcAg showed lower AUC values. The ROC analysis for HBsAg at weeks 0, –12, and –24 revealed lower results across all time points (0.49, 0.2; 0.52, 0.21; and 0.51, 0.23; respectively). HBcAg showed an AUC value of [0.56, 0.2], only measured at week 0. All values for all time points are depicted in Fig. 3B.

DYNAMICS OF THE SIMs OVER TIME

To assess the dynamic changes in the cytokine levels over time, a heat map was created (Fig. 4 and Supporting Fig. S1). Over the total cohort, IFN-γ and IL-13 showed a > 1.4-fold change of median cytokine levels (–1.46 and 1.46, respectively). Within the relapse group, IL-1Ra, IFN-α2, IFN-γ, and IL-13 changed > 1.4-fold (5.93, 4.36, –1.58, and 1.48, respectively), whereas in the nonrelapse group, > 1.4-fold changes were observed for LIF (2.09) and IL-8 (1.64). All SIMs previously identified as possible predictors of early VR with an AUC

of > 0.6 had fold changes of less than 1.2 in the entire cohort over the three time points, except for PDGF-BB (1.24).

COMBINATION OF SIM AND PREDICTION OF EARLY VR

Because single SIM measurement showed overall low AUC values of < 0.7, we analyzed a combination of cytokines to improve prediction of early relapse, applying a ML algorithm to identify the SIM combination with the highest predictive value. Due to the very uneven distribution of relapse in the TDF-treated group (only 2 nonrelapsers), no sufficient data were available in these patients. Thus, we focused on patients treated with ETV in further analyses, to find a solid and reliable biomarker to predict early VR.

First, we assessed the correlation among the single SIMs and their correlation with early relapse over the different time points (Fig. 5 and Supporting Fig. S2). Overall, the correlation of the specific SIMs with early VRR varied between $r = 0.01$ and $r = 0.39$. Almost all SIMs correlated strongly with at least one additional SIM at all three time points, and therefore delivered similar information regarding the outcome of early VR. This result suggests that subsets of SIMs contain
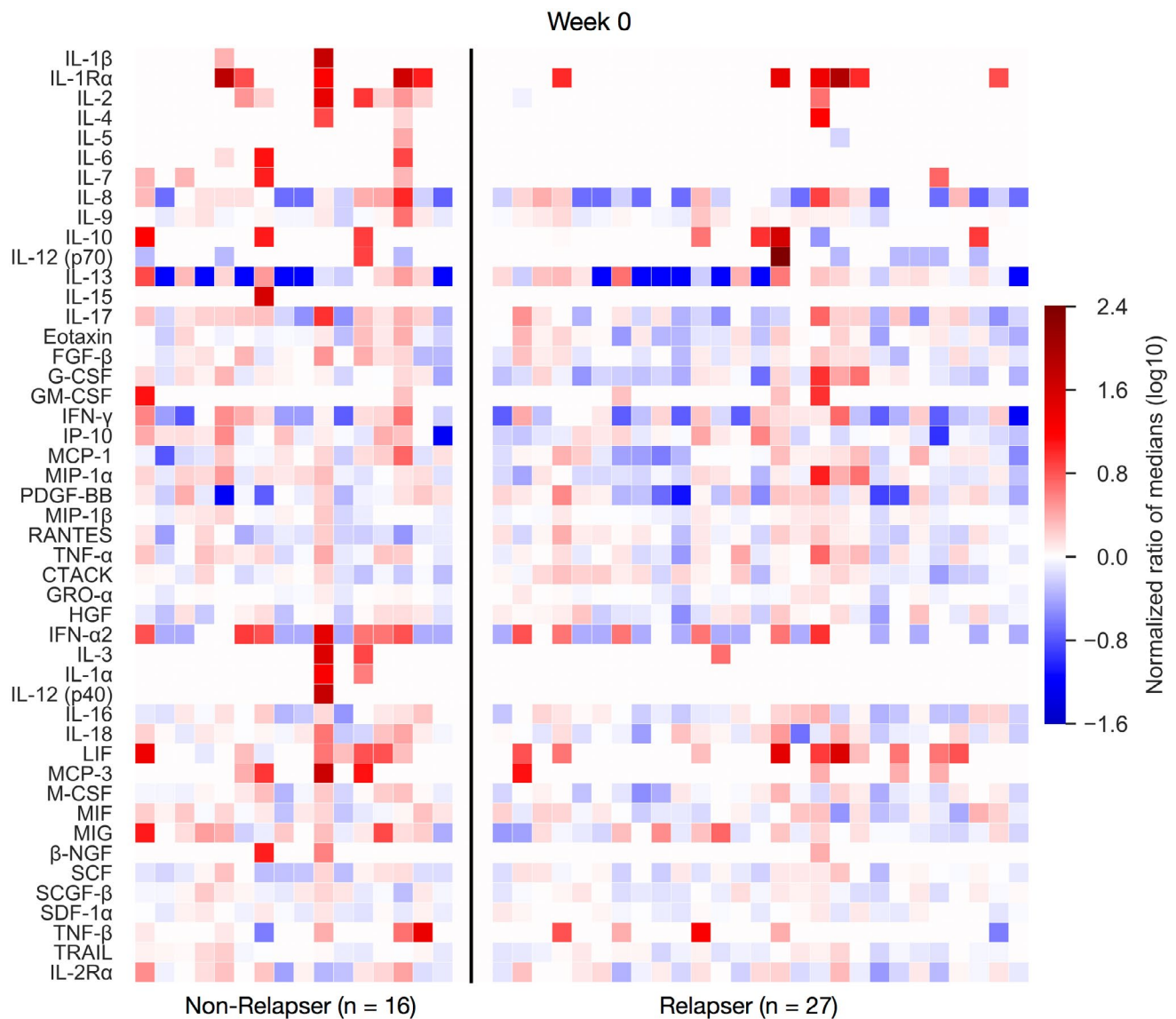
FIG. 2. Heat map distribution of the SIM. The heat map shows the expression levels of the 47 SIM for relapsers (n = 16) and nonrelapsers (n = 27) at time point of NA discontinuation (week 0) normalized by setting median = 1.

redundant information on the relapse probability, thus guiding us to reduce the dimensionality of the data set. The correlations for the time point of NA discontinuation (week 0) are shown in Fig. 5, while the correlations for weeks −12 and −24 are depicted in Supporting Fig. S2.

A supervised ML approach was implemented to figure out a combination of SIMs that gives us a high predictability for early VR at all three time points in the derivation cohort. This involves a systematic reduction of the SIM space to its most relevant

representatives, and connecting those to the smallest possible subset of SIMs with high predictive value for the three time points of the patient cohort. The feature combination with the highest predictive value at the time point of NA discontinuation was IL-2, MIG/CXCL9, RANTES/CCL5, SCF, and TRAIL.

At the time point of NA therapy withdrawal (week 0), this constellation of SIMs reached an AUC value of 0.89, 0.5-0.99 (Fig. 6A). Its predictability remained high at weeks −12 and −24 (0.76, 0.34-0.99; and 0.78, 0.1-0.99; respectively) (Fig. 6A).

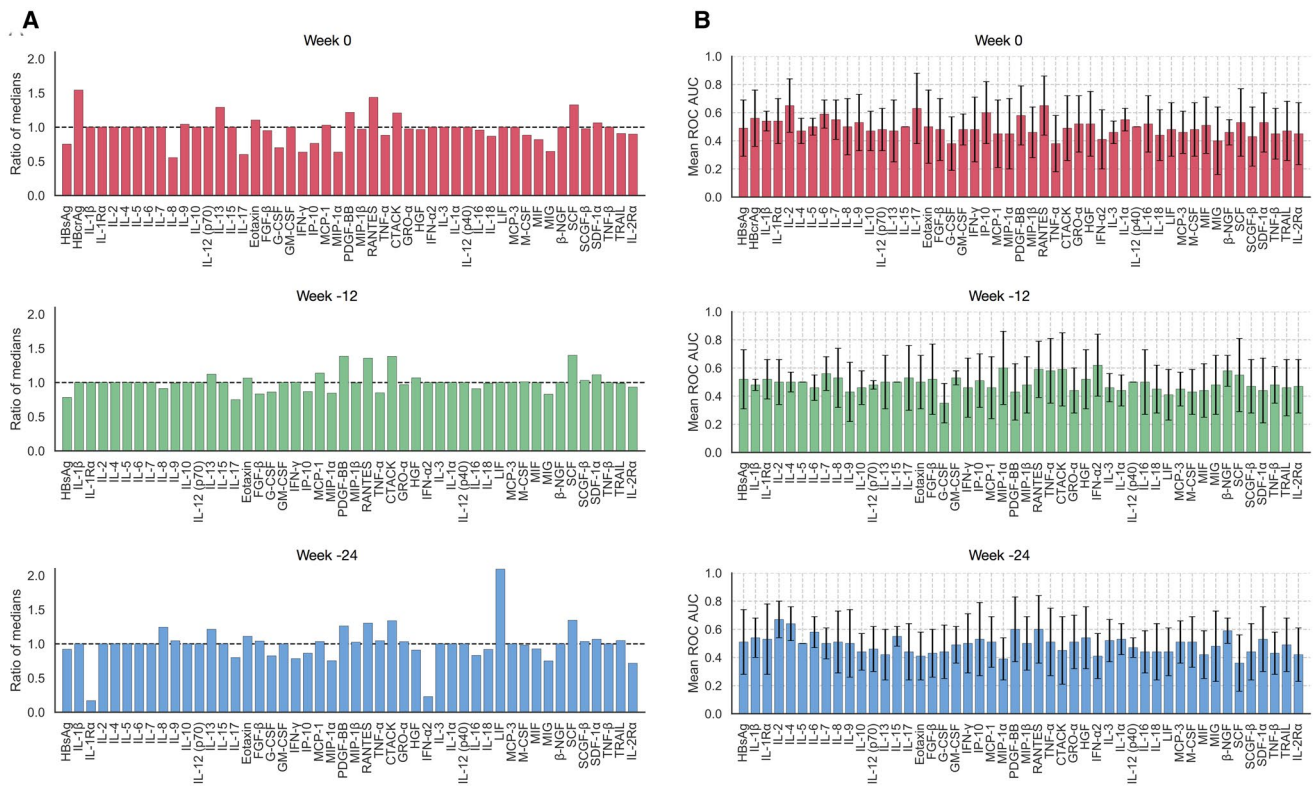**FIG. 3.** (A) Ratio (relapser/nonrelapser) of medians for respective SIM and viral marker at week 0 (red), week −12 (green), and week −24 (blue). The black line in the middle of each figure represents the value = 1 in which both groups have the exact same median. If the bar is higher than 1, relapsers have higher values; if the value is lower than 1, nonrelapsers have higher values. (B) AUC values (± SD) for the prediction of VR (HBV DNA > 2,000 IU/mL) for respective SIM and viral marker at week 0 (red), week −12 (green), and week −24 (blue).

Of note, the constellation of SIMs also showed good results at all three time points in the entire cohort, including TDF-treated patients (Supporting Fig. S3).

As previous studies have suggested a possible predictive role for HBsAg and HBcAg, we included these parameters as viral markers in our analysis. The predictive power of HBsAg, IL-2, RANTES/CCL5, SCF, and TRAIL at the time of cessation of NA treatment (week 0) was 0.76, 0.1-0.99, which is lower compared with the results with the five SIMs alone (Fig. 6C). However looking at week −12 and week −24 SIMs together with HBsAg had comparable AUC levels with the five SIMs alone (0.73, 0.17-0.99; and 0.81, 0.34-0.99; respectively) (Fig. 6C). A combination of HBcAg, IL-2, RANTES/CCL5, SCF, and TRAIL reached an AUC of 0.72, 0.1-0.99 at NA cessation (Fig. 6E). We conclude that the inclusion of viral markers into the set of predictive cytokine biomarkers does not provide any increase in the predictive power.

VALIDATION OF SIM COMBINATION IN AN INDEPENDENT COHORT

Next, we validated the combination of SIMs in an independent cohort of HBeAg-negative patients with chronic hepatitis B treated with ETV before treatment discontinuation (validation cohort). The cohort consisted of 49 patients (details are found in Table 2). The cohorts were different for the frequency of relapse within 24 weeks, gender distribution, and HBcAg levels (P = 0.038, P = 0.036, and P = 0.015, respectively), although HBcAg was low in both cohorts and at the lower limit of quantification (2.8 and 3.1 log U/mL) (Table 2). No significant differences in terms of age, HBsAg levels, ALT, and platelets were observed at the time point of treatment discontinuation (Table 2).

The five selected SIMs (IL-2, MIG/CXCL9, RANTES/CCL5, SCF, and TRAIL) reached an AUC of 0.63, 0.1-0.99 in the validation cohort (Fig.
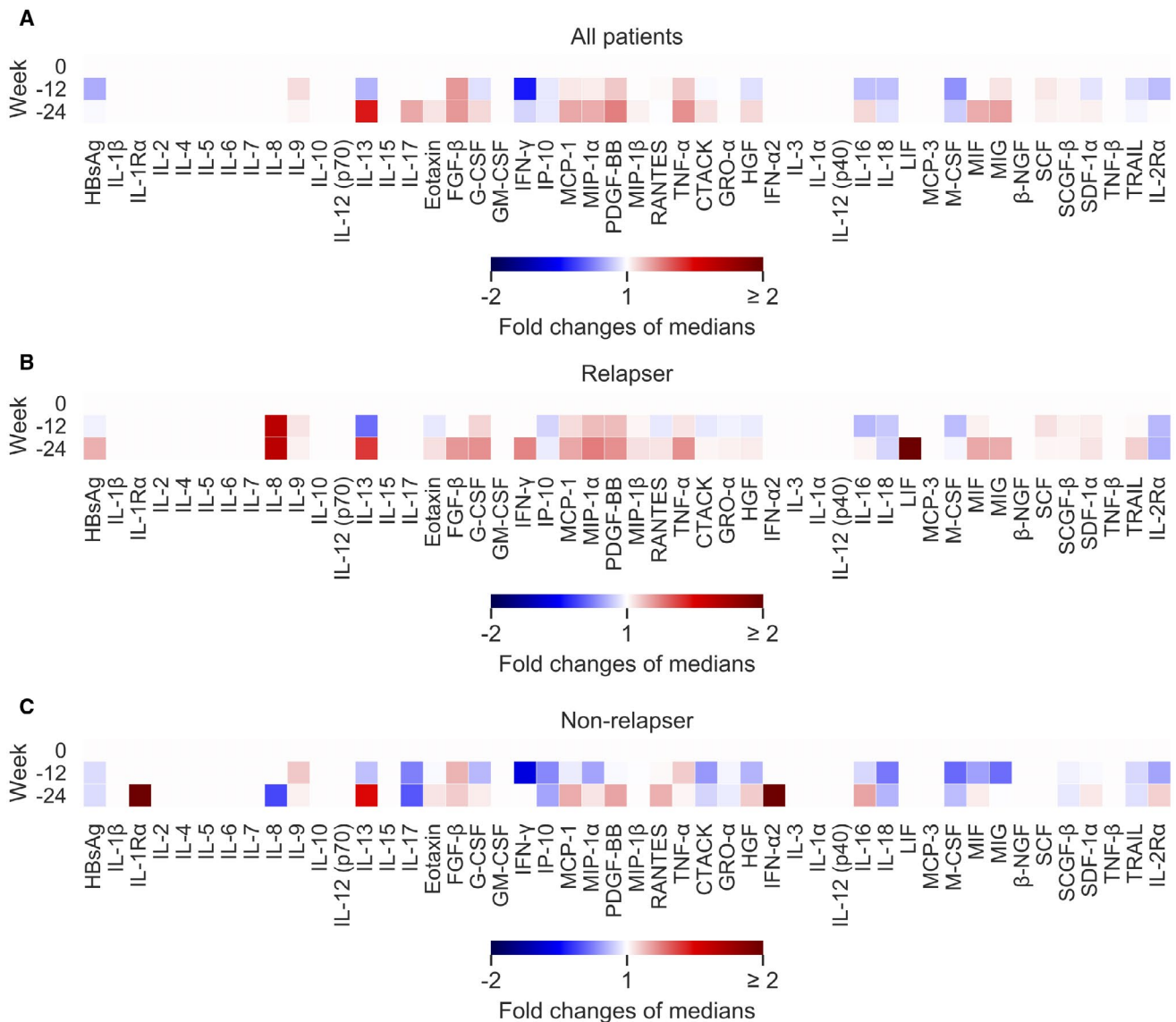
FIG. 4. Heat map showing the fold change of medians for 47 soluble immune markers and HBsAg before NA discontinuation for all patients (A), patients with VR (HBV DNA > 2,000 IU/mL) (B), and patients without VR within 24 weeks of follow-up (C). The time point of NA cessation (week 0) was set as standard.

6B). Adding the viral parameter HBsAg to the four cytokines (IL-2, RANTES/CCL5, SCF, and TRAIL), the predictive power was 0.59, 0.23-0.89 (Fig. 6D). Adding HBcAg to the four SIMs (IL-2, RANTES/CCL5, SCF, and TRAIL), the AUC was 0.67, 0.42-0.85 (Fig. 6F).

As HBsAg and HBcAg are currently the best evaluated viral markers to predict virological relapse, we tested their predictive power in the ETV group of the derivation cohort as well as in the validation cohort, to compare them with the SIMs. At the time point of

NA discontinuation, the AUC values for HBsAg and HBcAg were 0.56, 0.0-1.0 (validation cohort); 0.57, 0.2-0.8 (derivation cohort) and 0.59, 0.0-0.8 (validation cohort); 0.56, 0.0-0.9 (derivation cohort); respectively (Supporting Fig. S4).

Discussion

Stopping NA therapy in HBeAg-negative individuals remains a controversial topic. Although the AASLD

FIG. 5. Correlation of SIM with each other and with VR (HBV DNA > 2,000 IU/mL) at week 0. The second row shows the correlation of the combination of five SIMs, which demonstrated the highest AUC among all 47 cytokines for prediction of VR at the time point of NA cessation (week 0).
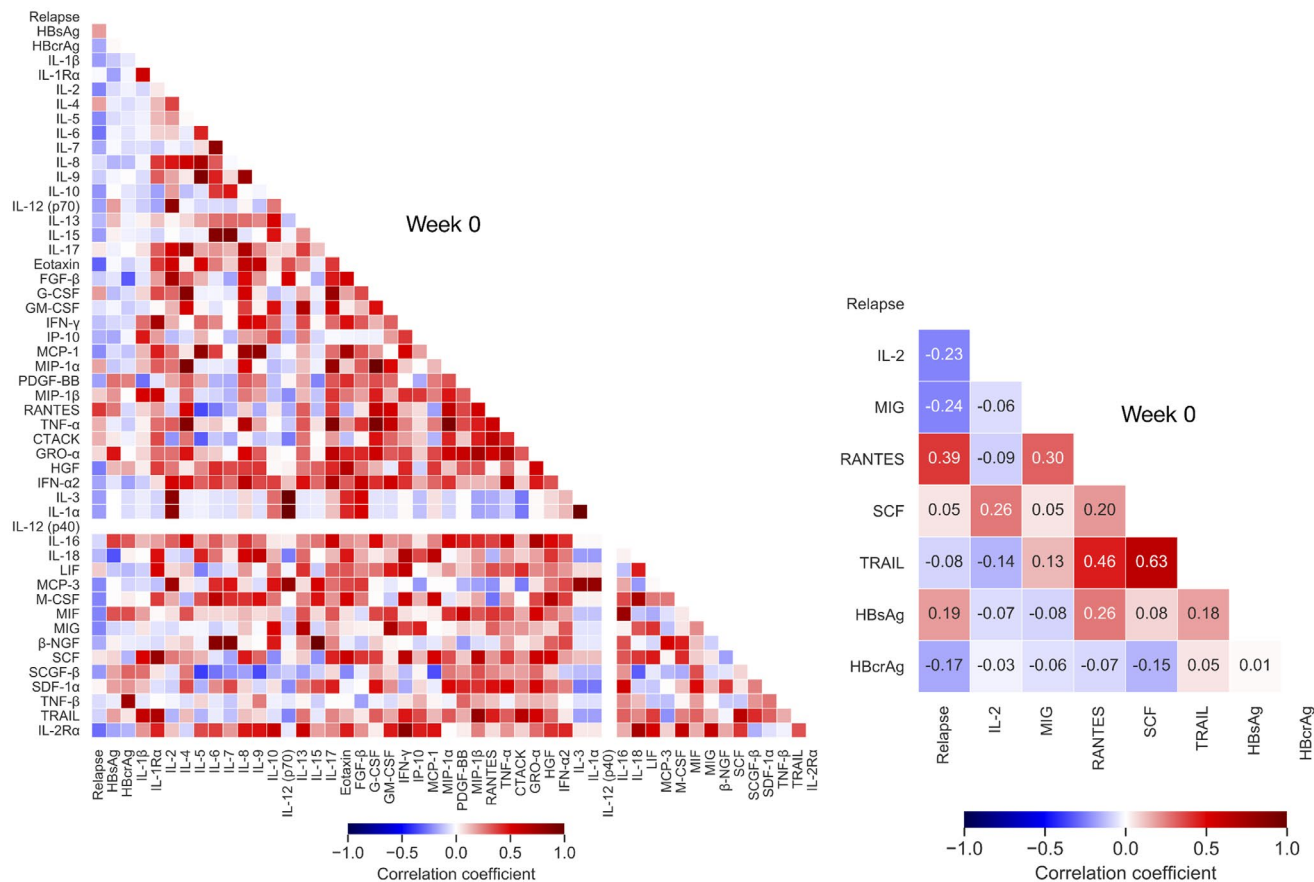
guideline does not recommend stopping NA therapy before HBsAg loss,[28] EASL as well as APASL guidelines consider stopping NA therapy in selected HBeAg-negative patients if a certain duration of consolidation therapy without detectable levels of HBV DNA was achieved (3 and 1-2 years, respectively).[2,7] Noteworthy, a reliable biomarker to predict VR is not yet established. While most studies have focused on viral parameters,[10-13,16,29,30] we investigated SIMs such as cytokines as host parameters, to find a reliable predictor for early VR, defined as HBV DNA > 2,000 IU/mL until week 24 after cessation of NA therapy. Although the overall number of patients is small, to our knowledge this is the largest cohort analyzing 47 SIMs at several time points before stopping NA therapy, thus providing in-depth insight into the cytokine milieu in this setting.

We show that (1) the cytokine profile is significantly different between patients with and without subsequent early relapse; (2) the performance of single SIMs for the prediction of early virological relapse is poor, but (3) a combination of five SIMs identified by ML is predictive for early VR; and (4) the identified SIM combination was tested in an independent cohort.

VR after stopping NA therapy occurs in most patients.[8] In our study, 63% experienced an early VR, defined as HBV DNA > 2,000 IU/mL until week 24 after cessation of treatment. Those patients who did not experience VR may control HBV DNA by immune responses. As shown by Rivino et al., immune responses such as programmed death-1–positive CD8+ T-cell responses may contribute to the control of HBV DNA and control viremia after stopping therapy.[31] Thus, the host immune marker might be a promising biomarker to predict relapse or virological remission. However, cellular immune responses
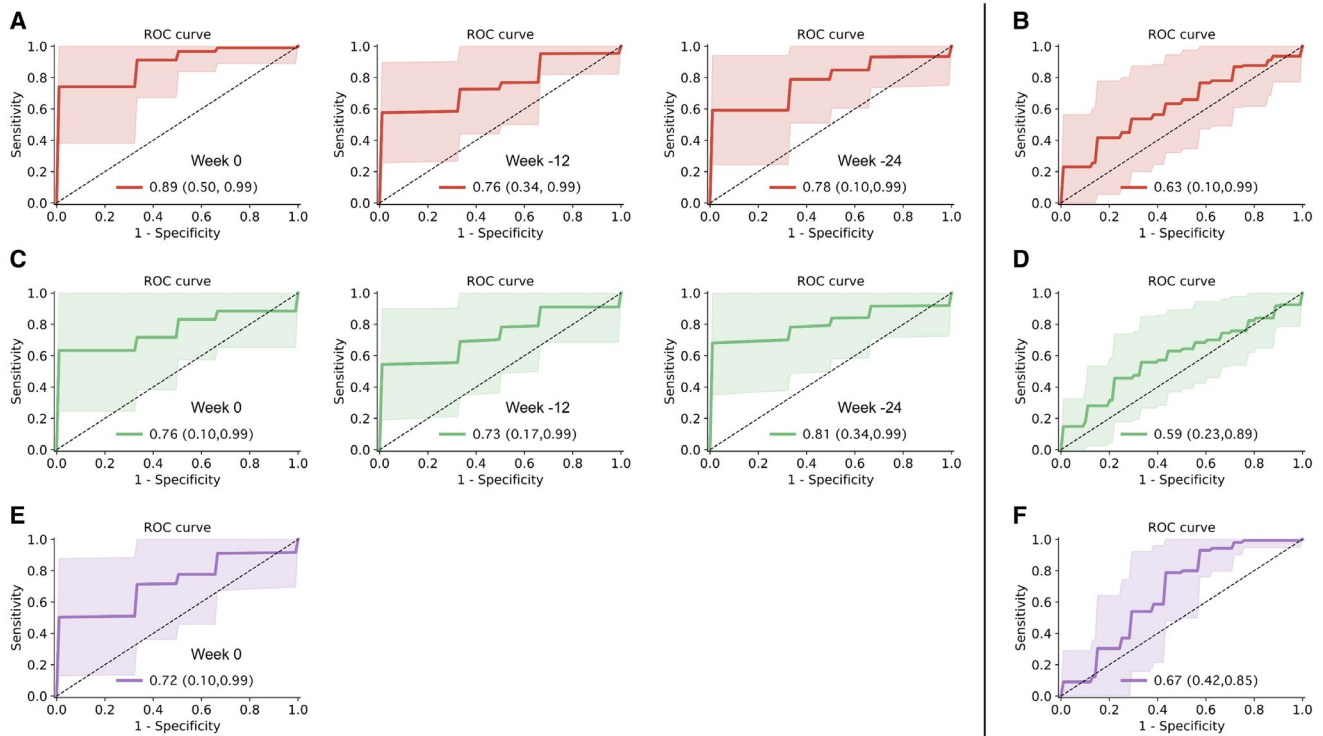
FIG. 6. Sensitivity and specificity for the best SIM combinations to predict VR (HBV DNA ≥ 2,000 IU/mL) identified by ML. Sensitivity and specificity of the five cytokines (IL-2, MIG, RANTES, SCF, and TRAIL) at weeks 0, –12, and –24 in the derivation cohort (A); the five cytokines (IL-2, MIG, RANTES, SCF and TRAIL) at the time point of NA discontinuation in the validation cohort (B); the four cytokines (IL-2, RANTES, SCF, and TRAIL) and HBsAg at weeks 0, –12, and –24 in the derivation cohort (C); the four cytokines (IL-2, RANTES, SCF, and TRAIL) and HBsAg at time point of NA discontinuation in the validation cohort (D); the four cytokines (IL-2, RANTES, SCF, and TRAIL) and HBcAg at week 0 in the derivation cohort (E); and the four cytokines (IL-2, RANTES, SCF, and TRAIL) and HBcAg at time point of NA discontinuation in the validation cohort (F). AUC, standard deviation and CI are provided in the respective graphs.

such as T cells are difficult to implement as biomarkers. SIMs measured in plasma or serum are much easier to implement. Recently, we have shown that SIMs differ in the different phases of chronic HBV infection, suggesting that SIMs are associated with control of HBV DNA.[32]

In our study, we identified a combination of five SIMs, including IL-2, MIG, RANTES, SCF, and TRAIL, which showed the highest predictive values for early VR at all three different points analyzed before NA cessation. In the overall derivation cohort, IL-2, MIG/CXCL9, and TRAIL tended to show higher results in the nonrelapser group, whereas RANTES/CCL5 and SCF had higher values in the relapse group. Interestingly, three of the five predictive SIMs, namely, IL-2, RANTES/CCL5, and MIG/CXCL9, target T-cell responses directly. IL-2 is an important cytokine associated with protective immunity, and the major source and the major target are activated T cells.[33] For HBV infection, it has also been shown that IL-2 down-regulates HBV gene expression in transgenic mice.[34] The main producer of RANTES/CCL5 is, again, T cells. The receptor CXCR3 (chemokine [C-X-C motif] receptor 3) is expressed on T cells and monocytes, and is associated with recruitment of these cells to infected tissue.[35]

The higher results of MIG/CXCL9 in the nonrelapser group indicate that IFN-γ responses may contribute to the control of HBV DNA after stopping NA. MIG/CXCL9 provides a measure of bio-active IFN-γ and a functional IFN-γ signaling pathway, and is important for immune cell migration.[36] Interestingly, in the transgenic mouse model, MIG/CXCL9 was produced by Kupffer cells, leading to a recruitment of further inflammatory cells to the liver.[37]

TABLE 2. BASELINE CHARACTERISTICS OF THE DERIVATION COHORT (MHH COHORT; n = 28) AND THE VALIDATION COHORT (CGMH COHORT; n = 49) AT THE TIME POINT OF NA DISCONTINUATION

Parameters	ABX Cohort (Derivation Cohort)	CGMH Cohort (Validation Cohort)	P Value
No. of patients	28	49	
Relapsers (until week 24)	14 (50%)	13 (26.5%)	0.038
Age (years)	58 (42-65)	57 (34-72)	0.836
Sex			0.036
Female	12 (42.9%)	10 (20.4%)	
Male	26 (57.1%)	39 (79.6%)	
ALT (U/L)	20 (12-38)	20 (12-44)	0.679
HBcAg log (U/mL)	2.8 (2.0-4.4)	3.1 (2.0-5.4)	0.015
HBsAg (U/L)	838 (6-12,971)	847 (4-19,382)	0.894
Platelets (/nL)	201.5 (105-385)	187 (117-317)	0.838

Note: Continuous data are presented as the median plus range. VR is defined as HBV DNA increase ≥ 2,000 IU/mL within 24 weeks after NA cessation.

The SCF receptor c-kit is up-regulated in diseased livers, suggesting an involvement in hepatic repair mechanism. Increased ckit messenger RNA expression was also observed in patients with liver failure.[38]

The level of TRAIL in nonrelapsers is more puzzling. TRAIL-expressing natural killer (NK) cells may control activated CD8+ T cells in HBV infection,[39] and it has been shown that NK-cell depletion, as well as TRAIL and NKG2D (NK group 2, member D) pathway blockade, induced a significant improvement of the HBV-specific T-cell function.[40] However, TRAIL expression on NK cells and soluble TRAIL level in the blood may not be related. It could also be an indicator of more apoptosis and control of HBV-infected hepatocytes. However, SIM levels were not distinctly different, and the balance and network with other SIM might be more relevant.

It is important to note that the supervised ML algorithm is looking for the highest predictive power for a SIM and may not necessarily identify SIMs that are biologically active themselves, but are strongly correlated with active cytokines, indicated by the strong predictive power. Several other cytokines previously linked with control of HBV infection, such IL-6, IL-1β, IP-10/CXCL10 and MIP-1α/CCL3, have emerged as being associated with early VR in the present cohort, but they failed to reach the most significant niveau and were therefore not included

as predictive markers, but might still be biologically active *in vivo*.

Importantly, our results could be validated to some degree in an independent cohort (0.63 [0.10-0.99]). Although the AUC is weak and the CI is high, it delivers results that are comparable to current viral markers, such as HBcAg and HBsAg. Differences in the AUC may be caused by differences between the cohorts. The patients from the validation cohort were more likely to be male, have higher HBcAg levels, and be nonrelapsers. In addition, we may have missed cofounders that were not assessed, such as HBV genotype, treatment duration, and type of infection. It should be noted that the frequency of relapse may align on further follow-up, and that the overall level of HBcAg of log 2.8 and log 3.1 U/mL is very low. It is also important to note that despite similar AUC, the addition of the viral marker HBcAg improved the CI when added to the SIMs, thus strengthening its reliability, which is poor if only the SIMs are considered (0.63, 0.1-0.99 and 0.67, 0.42-0.85, respectively).

Our study has some important limitations. First, the design of the study and the predefined follow-up of 24 weeks did not allow us to assess clinical relapse and long-term effects such as HBsAg loss. Although most patients experience early VR within 6 months, a certain number of patients relapse between 6 and 12 months or even between 12 and 24 months. However, the aim of this pilot trial was to show the general feasibility of ML in using SIMs as a predictor for early VR in HBeAg-negative patients who discontinue NA therapy. It also might be important to identify the patients who experience early VR, as it may subsequently lead to early clinical relapse for which a follow-up strategy could be tailored, for safety concerns. Recently, it has been shown that 19%-39% of patients may achieve HBsAg loss several months or years after stopping NA therapy.[19,41,42] In future studies, it would be very interesting to investigate whether a combination of SIMs could not only work in a long-term follow-up cohort, but also help to predict HBsAg loss after stopping NA therapy. Second, the handling of the serum samples between the derivation (frozen and shipped to Europe) and the validation cohort (measured locally) were different, possibly influencing cytokine levels. Additionally, the derivation cohort is based on a multicenter study that included patients across Southeast Asia and Oceania, whereas the validation cohort is from Taiwan and

reflects a possibly more homogenous study group. HBV genotypes were not assessed and may be different. Also, data on antiviral resistance to ETV or TDF are not available. Differences in HBV genotypes, type of infection (perinatal vs. acquired), and duration of NA therapy might be the reason for different relapse rates between the derivation and validation cohorts. Third, the data are derived from the Asia Pacific region; therefore, transferability of the results to other populations is limited. Genetic and environmental factors may influence immune responses[43,44]; thus, our set of SIMs may not be predictive in other settings. Given the AUC, the CI and potential differences between the derivation and validation cohort generalizability needs to be proved in further studies. Nevertheless, we clearly show that the approach to identifying a host biomarker based on a ML approach is possible. Importantly, we show that key SIMs and the predictive power of the identified SIM panel remain relatively stable 24 and 12 weeks before NA withdrawal, despite presumably changing cytokine levels in response to other environmental challenges, such as seasonal viral infections. Another important result of our study is that SIMs may have a better performance in predicting early VR than the viral markers HBsAg or HBcAg alone. However, a combination of SIMs and viral markers may be more reliable when comparing different cohorts.

In summary, we demonstrate that a combination of five SIMs as host markers is well-predictive of early VR, defined as HBV DNA > 2,000 IU/mL until week 24 after NA discontinuation in HBeAg-negative patients with chronic hepatitis B, and may be a useful addition to the viral predictors HBsAg and HBcAg. Supervised ML is helpful in deciphering the complex network of SIMs for the identification of an individual biomarker panel. Further studies should investigate the host immune marker as a biomarker for the individualized management of patients with chronic hepatitis B. Artificial intelligence will aid in identifying biomarkers in an unbiased approach.

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