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Longitudinalimmune phenotype assessment and serological outcome in foreign-born children with chronic hepatitis B

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ABSTRACT

OBJECTIVES. To assess changes in clinical phenotype, and identify determinants of outcome in children with chronic hepatitis B (CHB) virus (HBV) infection born in HBV-endemic countries followed in two Italian tertiary care centers after immigration or adoption.

METHODS. A prospective observational study on hepatitis B e antibodies (HBeAb)-negative CHB children started on 2002. Patients with liver fibrosis, or those needing antiviral treatment were excluded. *Immune active* patients were defined those with raised transaminases (ALT > 40 IU/L), *immune tolerants* those having normal ALT, both exhibiting substantial viral replication (HBVDNA > 2000 IU/ml).

RESULTS. Sixty-nine patients (44 boys, median age 4.7 years) had a median follow-up of 53 months. At entry, 18 (26%) children were immune tolerant, 47 (68%) immune active, and 4 had indeterminant immune status. At last follow up, 14 (78%) of the immune tolerant patients remained so, while only 23 (49%) of the immune active children maintained their initial immune phenotype. Seroconversion to HBeAb (SCHBe) occurred in only 2 (11%) immune tolerants, while 13 (28%) immune active patients achieved SCHBe.

Ethnicity was the only feature independently correlated to SCHBe: Asian origin reduced by 4.1 times the probability of SCHBe [Asian vs other; OR = 0.24 (95%CI = 0.07-0.76); $P = 0.016$] compared to other ethnicities, while viral genotype did not influence the outcome.

CONCLUSIONS. Ethnicity and immune status phenotype against HBV, rather than HBV genotype, are the main determinants of SCHBe in foreign-born children with chronic HBV infection.

Keywords: “hepatitis B”; phenotype; “immune tolerant”; “immune active”; seroconversion.

What is known

- Children's immune status against hepatitis B virus changes over time in chronic infection, its impact on outcomes being poorly understood.
- Asian children have been reported to be less susceptible to spontaneous seroconversion to hepatitis B e antibodies (HBeAb), but it is unclear whether that is due to ethnicity itself or to viral factors such as different genotype prevalence.

What is new

- Immune phenotypes behave differently over time, immune tolerant children largely remaining permissive to viral replication and immune active patients exhibiting a variable fate towards immune-clearance or chronic hepatitis
- Ethnic rather than viral factors influence HBeAb seroconversion in children born in endemic countries

Infographic: http://links.lww.com/MPG/B861

INTRODUCTION

Chronic hepatitis B (CHB) virus (HBV) infection is known to have a variable course, with different immune activity influencing viral replication and liver disease (1, 2). Although CHB has been considered a rather benign disease in pediatric age, 7-10% of the affected children develop severe hepatitis and 1-4% eventually develop hepatocellular carcinoma (3-5). Since long-term outcomes and indications to treatment are debated, defining determinants of outcome for CHB is of great importance.

Spontaneous or treatment-induced hepatitis B e antigen (HBeAg) seroconversion (SCHBe) is crucial for infection control, and is associated with fewer hepatitis events and overall a benign course (6). Studies in selected populations have identified predictive factors of spontaneous SCHBe, such as interleukin-10 and -12 production, pubertal stage, basal hepatitis B core antibodies (HBcAb), and higher transaminase levels (6-10). Efforts to understand the impact of HBV genotype on CHB outcomes have attained more conflicting results, due to the difficulty to dissect viral from host and environmental factors (11).

Moreover, even with the existing barriers to its implementation also in industrialized countries (12), large-scale immunization programs have brought changes in the epidemiology of HBV, thus historical data might not necessarily represent the current pediatric CHB population, mostly made of foreign-born children coming from endemic countries.

Beside the aforementioned viral, host and environmental factors, the categorization of patients with HBV infection according to clinical phenotypes reflecting immune status against the virus has been increasingly refined, in order to harmonize population data, estimate the need for antiviral treatment, and possibly predict the outcome of the infection (13). In fact, children with

an immune tolerant phenotype, albeit at less risk of hepatitis events and liver fibrosis in the short term, could bear an immunologic substrate favoring viral replication and integration likely to be associated with hepatocarcinogenesis on the long run, as suggested by epidemiologic studies (14). In adults, immune tolerant patients have been described to have a higher risk of hepatocellular carcinoma than treated immune active ones (15). In this context, in addition to the acknowledged indications to treatment of immune active patients with persistent marked transaminase elevation to prevent liver disease progression, an antiviral treatment could have a valuable role in counteracting the background of tolerance (16, 17). More recently, the EASL guidelines have emphasized the concept of the interchangeability of these chronic states one into another, proposing a new nomenclature based on "phases" (18).

Aim of our study was to longitudinally assess variations in clinical phenotype, to evaluate the clinical and serologic outcome and to identify determinants of outcome in children with CHB born in HBV-endemic countries, who were followed in two Italian tertiary centers after immigration or adoption.

METHODS

Patients

A prospective study was started in 2002 to evaluate the HBV phenotype of patients referred for CHB to the Hepatology, Gastroenterology and Transplantation Unit of the Hospital Papa Giovanni XXIII, Bergamo, and to the Pediatric Infectious Disease Unit of the Hospital L. Sacco, Milan.

Patients included in the database had age between 6 months and 18 years, had detectable serum hepatitis B surface antigen (HBsAg) for ≥ 6 months, and undetectable serum antibodies against HBeAg (HBeAb) and HBsAg (HBaAb).

At the time of the analysis patients were excluded in case of: history of hepatic decompensation or advanced fibrosis (judged by clinical features, liver Doppler ultrasound and platelet count), suspected or confirmed hepatocellular carcinoma, previous, ongoing or expected antiviral treatment, HIV and HCV coinfections.

Patients were defined as asymptomatic in absence of hepatomegaly.

Definitions and laboratory methods

Clinical immune phenotypes were defined according to the following criteria: *i)* immune active: ALT above the upper limit of normal (ULN) in at least one of two consecutive determinations 3-6 months apart; *ii)* immune tolerant: ALT below the ULN in two consecutive determinations 3-6 months apart; *iii)* inactive carriers: presence of serum HBeAb, absence of serum HBeAg, plasma HBVDNA < Log 4 IU/ml, and ALT below the ULN; *iv)* indeterminant: all HBeAb-negative patients exhibiting HBVDNA < Log 3.3 (~ 2000) IU/ml regardless of ALT level + all HBeAb-positive patients with plasma HBVDNA ≥ Log 4 IU/ml, and ALT above the ULN.

According to the local laboratory methods and reference range, 40 IU/L was considered the ULN for ALT.

Children were followed with outpatient visits every 6-12 months, and received laboratory tests assessing HBV serology, viral load and liver function tests every 3 to 6 months. The following clinical and demographic data were collected from the local electronic database: age at entry and last follow up, origin, family status (foster vs natural parents), mode of transmission, other

comorbidities, anthropometric data. Mode of transmission was classified as horizontal in presence of previous documented negative serum HBsAg and HBVDNA, while in absence of such information it was supposed to be vertical. The following laboratory data were collected: AST, ALT, GGT, total/direct bilirubin, albumin, PT INR, aPTT ratio, fibrinogen, complete blood cell count, alpha-fetoprotein, serum HBVDNA, HBsAg, serum HBsAb, HBeAg, HBeAb, total HBcAb, HBcAb IgM, viral genotype at study entry.

Persistently elevated ALT was defined as ALT above the ULN for ≥ 6 months and at least in 3 consecutive measurements.

HBVDNA was determined according to the locally used molecular methods. HBVDNA was expressed as $\log_{10}$ (Log) of viral genome IU/ml.

After automatic sample purification (QIAsymphony, QIAGENE), viral genotype was obtained by an in vitro assay for determining the DNA sequence of the reverse trascriptase (RT) of the Polymerase (Pol) gene of HBV (HBV sequencing, Abbott Diagnostics, Lake Forest, IL). Seqscape Software v 3.0 (ThermoFisher Scientific, Waltham, MA) was used to analyze the DNA sequencing data and consensus sequences have been sent to geno2pheno 2.0 web-based program to predict HBV genotype (https://hbv.geno2pheno.org, Max-Planck-Institut für Informatik, Saarbrücken, Germany).

The study was approved by the local Ethical Committees, and all parents/caregivers signed the approved study informed consent, as well as a signed assent was obtained from all children >12 years of age.

Statistical analysis

The Student t-test, the $\chi 2$ method, or Fisher's exact test were performed when appropriate for statistical analysis to compare continuous and categorical variables. Univariate logistic

regression was performed to analyze factors influencing SCHBe; statistically significant variables were then included in a multivariate model adjusted for covariates in order to identify independent risk factors. Kaplan-Meier analysis and Log-rank test were used for cumulative proportion curve comparison. A *P*-value < 0.05 was chosen as cut-off for significance. Data were analyzed with SPSS (IBM Corp. Released 2011. IBM SPSS Statistics for Mac, Version 20.0. Armonk, NY: IBM Corp) and GraphPad Prism (GraphPad Prism version 5.00 for Mac, GraphPad Software, San Diego, CA) softwares.

RESULTS

Out of 76 children with chronic HBV infection, 6 children were excluded because of HBeAb positivity, and one was excluded because he qualified for antiviral treatment. Sixty-nine patients (44 boys) were enrolled at a median age of 4.7 years (6 months-15.5 years) and followed for a median time of 53 (6-170) months. HBV transmission was supposed to be vertical in the vast majority. Fifty-six (81%) were adoptees, and all children were foreign-born, more than half being of Asian origin. No patient had HCV or HIV coinfection.

None of the patients included was cirrhotic as judged by ultrasound liver texture, spleen size and platelet count, and liver synthetic function was preserved in all.

Patients were divided in three groups, according to the HBV phenotype at study entry: 18 (26%) children were classified as immune tolerant, 47 (68%) as immune active, and 4 as indeterminant. The demographic, clinical, and laboratory features of the patient groups are displayed in Table 1. As expected, immune tolerant children had significantly lower transaminases with respect to the others, while those classified as indeterminant had lower viral load and were older in comparison

to immune tolerant and immune active patients. Ten patients were HBeAg-negative, 3 classified as immune tolerant, 3 as immune active and 4 being indeterminant.

Viral genotype was available for 50 patients (72%). Genotype B was more frequent among immune tolerant children than in immune active (57% vs 13%), whereas genotypes A and D were less common among immune tolerant than in immune active patients (0 and 43% vs 21% and 49%, respectively; $p = 0.001$).

Outcome of the HBV infection phenotype

Figure 1 summarizes patients' HBV infection outcome at study end. All the patients showing indeterminant phenotype achieved SCHBe shortly after the study entry, after a median time of 3.5 (range 3-23) months, and normalized transaminases, becoming inactive carriers. These four HBeAg-negative patients presenting with raised transaminases and low viral load were interpreted as already undergoing SCHBe, and were excluded from the subsequent analysis of the HBV phenotype impact on infection outcomes.

The majority (78%) of the immune tolerant patients remained so, while inactive carrier in two patients, and immune active and indeterminant status in one patient each were achieved. Conversely, less than a half (49%) of the immune active patients maintained the same phenotype, while 7 (15%) became inactive carriers, 6 (13%) became immune tolerants, and the others were classified as indeterminants (5 being HBeAb-negative/HBeAg-positive children with normal transaminases and low viremia, 6 having seroconverted to HBeAb but with still increased transaminases and viral load).

In Figure 2 (A, B), patients' phenotype at entry (Figure 2, A) and at study end (Figure 2, B) is plotted.

At the end of follow-up, all children had remained in good conditions, and none of them died nor had developed chronic liver disease or hepatocellular carcinoma; 39% of them had raised ALT. In all children with immune active hepatitis, hepatitis D virus (HDV) infection was excluded by specific antibody testing.

No SCHBs was observed, but 19/69 patients (27%) achieved SCHBe after a median time of 2.6 years (1 month - 12 years), with an overall SCHBe rate of 5.81%/year. The proportion of children achieving SCHBe was higher among those presenting as immune active at entry than in immune tolerant, but the difference did not reach significance (28% vs 11%, respectively; p = 0.160).

However, 68% of the patients achieving SCHBe also became inactive carriers, and reached significantly lower replication in comparison with those who remained HBeAb-negative (3.6 ± 1.9 vs 7.3 ± 2.1 HBVDNA Log IU/mL; p <0.001).

Clinical and laboratory features according to serologic outcome are displayed in Supplemental Table 1 (Supplemental Digital Content, http://links.lww.com/MPG/B860).

Determinants of SCHBe

Since the immune response against the HBeAg is crucial in HBV infection control, we looked for possible factors influencing SCHBe.

In Table 2 the role of demographic, clinical and laboratory features in determining SCHBe is investigated through univariate analysis. The rate of SCHBe was not associated with gender, nor with the occurrence of phases of persistent hypertransaminasemia, the age, the HBV infection phenotype and the HBVDNA at entry. However, Asian ethnicity was associated with a chance reduced by 4.6 to achieve SCHBe [Asian ethnicity vs SCHBe; OR = 0.21 (95%CI = 0.07-0.67);

P = 0.008], while no role of the HBV genotype in determining the SCHBe was observed. Also, the presence of hypertransaminasemia at entry was substantially correlated with SCHBe [OR = 4.00 (95%CI = 0.82-19.44); *P* = 0.086].

However, when pooled in a multivariate analysis with the presence of elevated ALT at entry, the Asian origin proved to independently prevent the occurrence of seroconversion [OR = 0.24 (95%CI = 0.07-0.76); *P* = 0.016], reducing by 4.1 the probability of seroconversion against HBe compared to other ethnicity.

Figure 2 (C, D) illustrates the survival curve comparison at Kaplan-Meier analysis for the occurrence of SCHBe according to origin and phenotype. The rate of SCHBe was significantly lower in Asian children (2.88%/year) compared to those of other (African, Caucasian) origin (10.7%/year; *p* = 0.006; Figure 2, C). On the other hand – as depicted in Figure 2, D – although the rate of SCHBe was lower in immune tolerant children (2.29%/year) than in immune active (7.47%/year; *p* = 0.202) this difference did not reach significance in our cohort.

DISCUSSION

The present study longitudinally assesses categorized clinical phenotypes in children with CHB. This study design provides a description of the dynamic behavior of such phenotypes in childhood. We observed that only 11% of patients classified as immune tolerant achieve the inactive carrier status (2.29%/year), whereas some 28% of the immune active children (7.47%/year) enter the immune-clearance phase through a SCHBe over a 5-year follow-up. This rate of spontaneous seroconversion to HBeAb is not far from that described by Hong in immune tolerant Korean HBV-infected children (15.8% in a 10-year follow-up) (19), while adult rates are

much higher (20). Unlike other studies emphasizing the role of the age on SCHBe (19, 21), in our cohort the phenotype marked a different trend towards significance.

Nowadays, immune tolerance in young patients is considered immunologically different from that of adults, characterized by high level of HBVDNA integration, clonal hepatocyte expansion with malignant potential, relatively preserved anti-HBV T-cell response (22). Despite the potential harm of a persistent tolerant status through young adulthood, AASLD guidelines recommend against antiviral treatment of such patients (2). Conversely the EASL recommends nucleot(s)ide analogues-based long-term viral suppression in immune tolerant patients ≥ 30 years of age (18), due to the increased risk of fibrosis and cirrhosis in patients with a delayed immune-clearance (20, 23).

Pediatric age window has been regarded as a good time to break the tolerance towards the virus, thus sequential protocols of interferon alpha and lamivudine or entecavir have been employed, with SCHBe ranging from 3% to 33% and SCHBs up to 22% in those utilizing interferon/lamivudine combination (17, 24, 25).

On the other hand, immune active children migrate towards inactive carrier state in a proportion as high as one third over a 5-years follow-up, the others remaining in a chronic hepatitis status with risk of liver disease progression.

In a scenario characterized by the lack of clear indications to treatment, and by disappointing therapeutic outcomes, a clear definition of the phenotypes has great importance to prognostic purposes and for patient selection in possible trials.

We used a definition of clinical phenotypes slightly different from that published by the Hepatitis B Research Network (13, 26). Namely, the chosen ALT cut-off to define hepatitis activity was 40 IU/mL, higher than that used in previous studies, but reflecting our Institution's ULN and

EASL definition (18). This could have accounted for the relatively high proportion of immune tolerant children in our cohort. Similarly, HBVDNA level to differentiate between immune tolerant/immune active and indeterminant was Log 3.3 (~ 2000) IU/mL HBVDNA, according to the definition of HBeAg+ or HBeAg- CHB and also representing the cut-off for viral suppressive treatment (18). Schwarz at al provided a cross-sectional description of a large pediatric cohort of 371 patients, with immune tolerant children being 12-35% depending on the ALT cut-off used, and a large proportion of indeterminant HBeAg-negative children with abnormal ALT and low HBVDNA (13).

Patients' allocation to phenotypes was also affected by ethnic origin, the latter ultimately proving to be the only factor associated with the occurrence of SCHBe. In fact, only 2.88%/year of the Asian group seroconverted in comparison with 10.77%/year of the remaining children.

Also Marx et al described a significantly lower rate of SCHBe in children of Asian origin living in the US, in comparison to the other ethnic groups, mostly Canadians and Eastern Europeans (75% vs 94%; $P < 0.05$) after 13 years of follow-up (27).

We did not find any role for the viral genotype in influencing the SCHBe, unlike other reports correlating genotypes C and D with the immune tolerant phenotype (13, 26), and genotypes B and C with a lesser likelihood of SCHBe (11, 28). However, genotype B was almost exclusively present in Asian children of our cohort. The fact that genotype was not assessed in all the study patients and that possible genotype changes have not been explored, could have affected the ability to evaluate virus-related outcome determinants. Also, the low numbers of patients for each genotype could have a role in the fact that the viral genotype was not decisive in determining patients' outcome.

In conclusion, we provided a natural history of HBeAg-positive children with CHB from the perspective of clinical phenotypes defining the immune status against the virus. We observed that only a small number of immune tolerant patients reach SCHBe, and that the majority of them show a persistent permissive behavior towards viral replication. On the other hand, immune active patients exhibit a much more diverse phenotypical fate over time, with one third of immune active children spontaneously clearing the HBeAg, the others changing towards possible transitional phenotypes or remaining at risk of organ failure and malignancy, potentially qualifying for antiviral treatments. Apart from the clinical phenotype, the other factor influencing serologic outcome is the ethnicity, and we demonstrated that this is a host-related factor independent from the viral genotype – a previously unaddressed issue in the pediatric population. Larger studies with longer observation after SCHBe are required to better define the real-life value of the phenotype and the impact of host factors in the categorization of CHB management.

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Figure legends

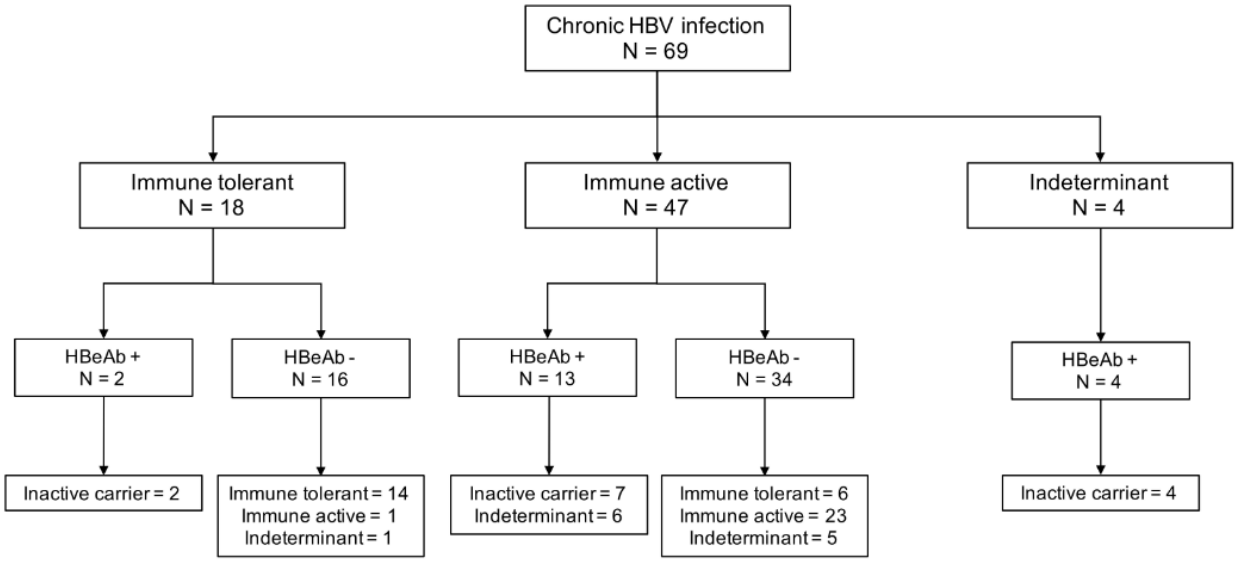

Figure 1. Outcome of 69 children with chronic hepatitis B according to the clinical phenotype.

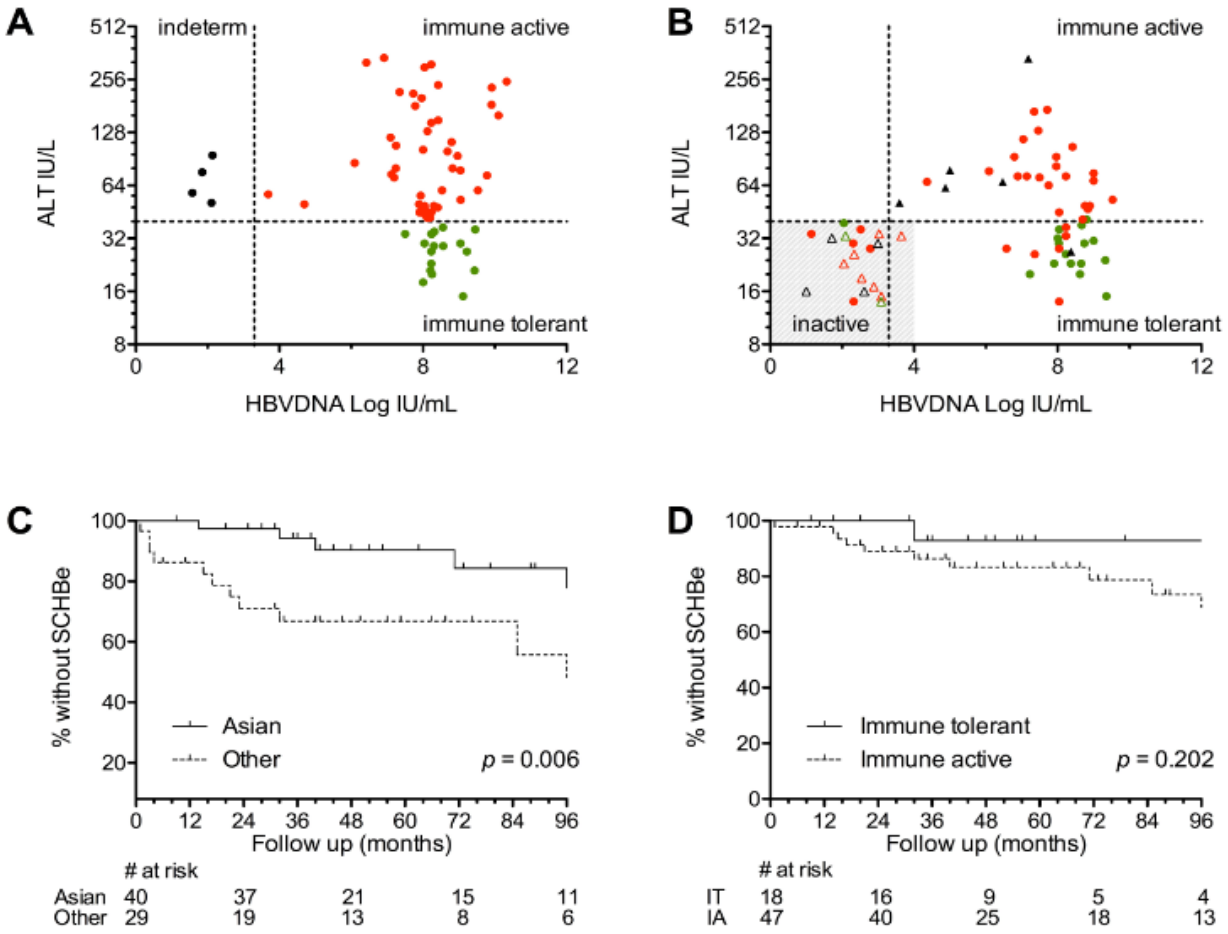

Figure 2. A, B) Scatter plot of the clinical phenotype of 69 patients with chronic hepatitis B at study entry (A) and at the end of the observation (B). In panel 2, A, immune active (red), immune tolerant (green), and indeterminant patients (black) are plotted. In panel 2, B, the symbols' color represents phenotype at entry [immune active (red), immune tolerant (green), and indeterminant patients (black)]; the triangles represent the patients that achieved seroconversion to HBeAb: the empty triangles are inactive carriers, the black-filled triangles are indeterminant. Also, HBeAb-negative patients plotted in the grey area are indeterminant.

C, D) Kaplan-Meier analysis and Log-rank comparison of the HBeAb seroconversion-free survival curves of children with chronic hepatitis B according to ethnic origin (C) and clinical phenotype (D).

Indeterm: indeterminant; SCHBe: seroconversion to HBeAb.