Early intrahepatic recurrence of HBV infection in liver transplant recipients despite antiviral prophylaxis

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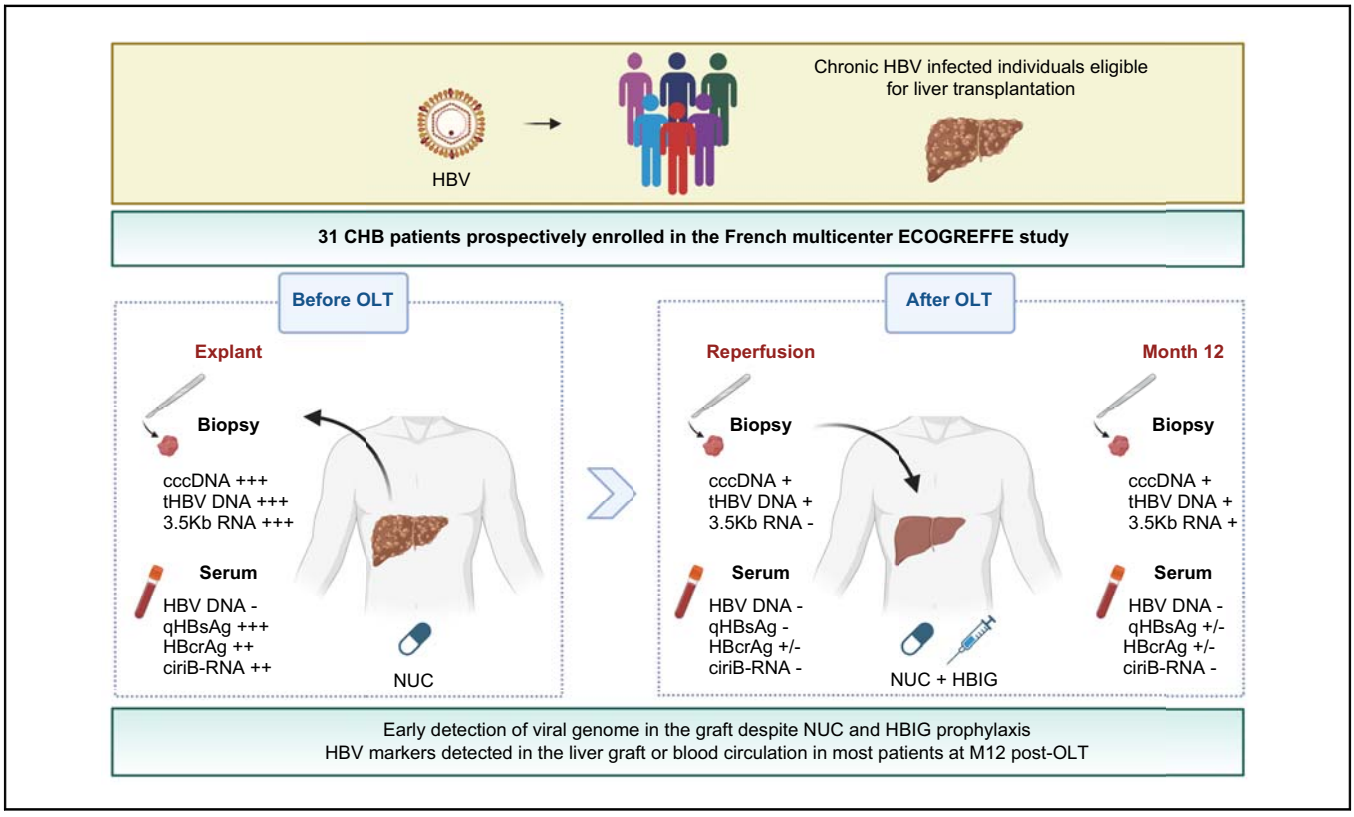
Graphical abstract



Highlights

- This is the first prospective real-life study investigating intrahepatic viral markers in patients who underwent a transplant.
- Viral genome was detected very early in the graft despite NUC and HBIG prophylaxis.
- At 12 months post OLT, HBV markers were detected in the liver graft or blood circulation in all but one patient with matched samples.

Impact and Implications

In this work, we show that, despite the recommended prophylaxis based on NUC and HBIG, HBV can infect the new liver very rapidly after transplantation. Twelve months after transplantation, the majority of patients had at least one HBV marker detected in either serum or the liver. Therefore, our results demonstrate early intrahepatic viral recurrence despite NUC and HBIG therapy and underline the importance of an optimal patient compliance to the antiviral prophylaxis to prevent viral rebound.

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Research article

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Early intrahepatic recurrence of HBV infection in liver transplant recipients despite antiviral prophylaxis

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Background & Aims: Prophylaxis with nucleos(t)ide analogues (NUCs) and hepatitis B immunoglobulin (HBIG) has decreased the rate of HBV recurrence after orthotopic liver transplantation (OLT), but the duration of this prophylaxis remains debated. Our aim was to investigate the recurrence of both intrahepatic and serum HBV markers after OLT in patients receiving long-term NUC and HBIG prophylaxis.

Methods: A total of 31 HBV-positive patients benefiting from OLT were prospectively enrolled in five French centres between 2012 and 2015. Tissue samples from the native liver, liver reperfusion biopsy, and 12-month post-OLT (M12) biopsy were collected. Intrahepatic HBV markers were quantified using Droplet Digital PCR. Serum hepatitis B core-related antigen (HBcrAg) and HBsAg were quantified using the Lumipulse platform.

Results: Among the 31 patients, 26 were HBeAg negative and 28 had undetectable serum HBV DNA at OLT. All patients received HBIG and NUC after OLT, and serum HBV DNA was undetectable at M12. Of the 27 available native livers, 26 had detectable total HBV DNA (median, 0.045 copies/cell), 21 were positive for cccDNA (0.001 copies/cell), and 19 were positive for 3.5-kb HBV RNA (0.0004 copies/cell). Among the 14 sequential reperfusion and M12 biopsies, seven were positive for HBV markers on the reperfusion sampling, and six of them were also positive at M12. Of the 27 patients with available serum samples at M12, eight were positive for HBcrAg and five were positive for HBsAg by ultrasensitive quantification, although they were negative by conventional techniques. Overall, among the 17 patients having a matched biopsy and serum sample at M12, only one had undetectable HBV markers in both the liver and serum.

Conclusions: Our results demonstrate a very early detection of viral genome in the graft and intrahepatic viral recurrence despite NUC and HBIG prophylaxis.

Clinical Trials Registration: This study is registered at ClinicalTrials.gov (NCT02602847).

Impact and Implications: In this work, we show that, despite the recommended prophylaxis based on NUC and HBIG, HBV can infect the new liver very rapidly after transplantation. Twelve months after transplantation, the majority of patients had at least one HBV marker detected in either serum or the liver. Therefore, our results demonstrate early intrahepatic viral recurrence despite NUC and HBIG therapy and underline the importance of an optimal patient compliance to the antiviral prophylaxis to prevent viral rebound.

Keywords: HBV; Liver transplantation; cccDNA; Viral kinetics; Disease recurrence; Biomarkers.

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Introduction

Chronic hepatitis B (CHB) affects 296 million people worldwide and is the main cause of hepatocellular carcinoma (HCC).[1] CHB infection represents a major cause of morbidity and mortality, owing to severe complications such as liver cirrhosis and HCC.[2] Current CHB treatments, based on nucleos(t)ide analogues (NUCs), are effective in decreasing viral load but are curative in less than 5% of cases. Orthotopic liver transplantation (OLT)

represents the definitive treatment for decompensated CHB cirrhosis, complicated or not by HCC. Currently, CHB is a common indication for OLT and represents 30% of all OLT performed in Asia and 10% in Europe and the USA.[3,4]

Historically, CHB was considered a contraindication owing to the high risk of HBV recurrence after OLT, resulting in graft losses and poor survival rate of less than 40% at 5 years.[5] The prophylaxis regimen used to prevent HBV recurrence has changed over the last three decades with the advent of hepatitis B immunoglobulins (HBIG) and different generations of NUC.[6,7] Currently, by combining third-generation NUC and HBIG perfusions, the rate of HBV recurrence after OLT, defined by HBsAg positivity in the serum, is less than 10%.[8,9] Thus, this combined prophylactic regimen is recommended by most international liver societies.[10–13] Because of the high cost of the administration of HBIG by i.v. infusion or s.c. injection, reducing the HBIG prophylaxis duration is considered in specific patient populations based on the exclusion of risk factors such as pre-OLT viral load, HDV coinfections and when optimal patient compliance to treatment is ensured.[13]

Several studies have reported the use of NUC monotherapy without HBIG for the prophylaxis of HBV recurrence. These studies reported a rate of detectable HBsAg in serum of approximately 8–10% after a follow-up of 2–8 years post OLT, which was associated with maintained viral suppression and the absence of virologic relapse when using NUCs with high barrier to resistance, and overall a very good survival rate.[14–16] In these patients, the detection of HBsAg indicates that NUC monotherapy does not fully prevent reinfection of the graft. Indeed, it has been shown in hepatocyte culture models that NUCs inhibit the viral replication but do not prevent either covalently closed circular DNA (cccDNA) formation in *de novo* infected hepatocytes or HBV genome integration and HBsAg production.[17,18]

None of the previously mentioned studies on simplified prophylaxis have investigated HBV intrahepatic markers, and only a few retrospective studies have analysed HBV recurrence on liver biopsies.[19] In a retrospective study of 12 patients with a 60-month follow-up, under double prophylaxis, cccDNA was quantifiable in nine of them (including 3/3 who had a quantifiable serum viral load at OLT).[20] In a study of 44 patients with undetectable serum viral load at 10 years post OLT, one patient had detectable total HBV DNA in the protocol biopsy.[21] Another investigation including 25 patients who underwent a transplant for HBV receiving dual NUC–HBIG prophylaxis has reported the detection of intrahepatic total (t)HBV DNA and cccDNA 3 years after OLT in 87 and 17% of samples, respectively, and a recurrence of serum HBsAg in two (8%) patients.[22] In another study including patients receiving lamivudine prophylaxis after OLT, two of 44 (4.5%) patients were positive for tHBV DNA but none for cccDNA after an average of 88 months; 56% of these patients had received HBIG prophylaxis.[23] In another retrospective study, 12/20 patients were positive for both cccDNA and serum hepatitis B core-related antigen (HBcrAg) after OLT, and the levels of both markers were positively correlated.[24] Overall, these studies were heterogeneous regarding the timing of viral markers analysis after OLT and did not assess the recurrence of HBV infection at early time points. Moreover, more sensitive quantification technologies such as Droplet Digital PCR (ddPCR) assays have been developed since then, which could allow a more precise appraisal of HBV recurrence on the graft.[25–28]

In this context, the primary objective of the ECOGREFFE prospective cohort study was to analyse HBV DNA (cccDNA and tHBV DNA) and RNA presence in the native liver and the graft with more sensitive assays to gain insight into the kinetics of HBV recurrence in patients receiving the recommended long-term NUC and HBIG prophylaxis in France. The secondary objective was to investigate the correlation between intrahepatic viral molecular markers and novel serum HBV biomarkers.

Patients and methods

Prospective cohort

Patients older than 18 years, with or without HCC, and listed for OLT were eligible for inclusion in the French prospective multicentre study 'ECOGREFFE' (clinical trial number: NCT02602847) between 2012 and 2015 in five French OLT centres: Lyon-Croix Rousse, Villejuif-Paul Brousse, Montpellier, Nice, and Grenoble. The present study is an ancillary study to the ECOGREFFE study. The inclusion criteria were as follows: being included in the ECOGREFFE study and having benefited from an OLT for an indication of HBV-related disease. Medical information and laboratory results were collected prospectively. Three patients with positive HDV serology were excluded from the analysis.

Whenever possible, a liver sample from the native HBV-infected liver (27 patients), from reperfusion biopsy (26 patients), and at 12 months after OLT (M12) (17 patients) were collected. In parallel, blood samples were collected at the time of registration on the OLT waiting list (30 patients), at M3 (29 patients), and at M12 (27 patients). In addition, according to the ECOGREFFE study protocol, six samples from patients who underwent a transplant for an alcoholic-related disease or HCV-related disease at the Lyon-Croix Rousse liver transplant centre were used as technical negative controls. Liver samples were snap frozen, and all serum and tissue samples were stored at -80 °C until processing. For each patient, the clinical follow-up duration was 12 months post OLT. The study was conducted in accordance with the relevant ethical standards and approved by the local advisory committee (IRB: CPP Sud-Est IV [ref.: A-12-158]; ID RCB: 2012-A00383-40).

Clinical virological tests

Conventional laboratory tests

Serum viral load was quantified in each centre using either the Roche assay (Roche Diagnostics, Mannheim, Germany) or the Abbott assay (Abbott Diagnostic, Chicago, IL, USA), with detection limits of 10 and 20 IU/ml, respectively. The conventional HBsAg quantification was carried out using the conventional methods in each centre using either the Roche assay (Roche Diagnostics) or the Abbott Architect assay (Abbott Diagnostic), with a limit of quantification set at 50 mIU/ml.

Ultrasensitive HBsAg quantification

Quantitative levels of HBsAg at OLT registration time, at M3, and at M12 were determined using the Lumipulse G HBsAg assay (Fujirebio Europe, Gent, Belgium) on the Lumipulse G600II analyser (Fujirebio) according to the manufacturer's instructions. HBsAg levels were expressed in mIU/ml, and the assay measurement linear range spanned from 5 to 150,000 mIU/ml. Samples for which the HBsAg level was <5 mIU/ml were considered negative. Samples for which the HBsAg level was ≥150,000 mIU/ml were diluted with a manufacturer-supplied dilution reagent and retested to quantify HBsAg values.

HBcrAg quantification

The serum HBcrAg quantification assay simultaneously measures the concentration of denatured HBeAg, HBcAg, and the precore protein p22cr (aa -28 to aa 150). The quantitative levels of HBcrAg from patients' sera were determined using the Lumipulse G HBcrAg assay (Fujirebio) on the Lumipulse G600II analyser (Fujirebio) according to the manufacturer's instructions. HBcrAg levels were expressed in logU/ml. The lowest sensitivity limit of the machine was 2 logU/ml, but a specificity of 100% is reached for values ≥2.8 logU/ml.[29] Therefore, samples for which the HBcrAg level was ≥2 and <2.8 logU/ml were considered negative. Samples for which the HBcrAg level was ≥2.8 and <3 logU/ml were considered detectable but not quantifiable. Samples for which the HBcrAg level was ≥7 logU/ml were diluted with a manufacturer-supplied dilution reagent and retested to quantify HBcrAg values.

cirB-RNA quantification

Circulating HBV RNA (cirB-RNA) was quantified by real-time PCR using the Roche HBV RNA investigational assay (Roche Diagnostics, Pleasanton, CA, USA) for use on the Cobas 6800/8800 Systems (Roche Diagnostics).[30] The assay is a nucleic acid quantitative test performed with EDTA plasma or serum sample, for which the lower level of quantification is 10 copies/ml (linearity range 10 to 10^9 copies/ml on an armoured RNA template) and the lower level of detection is three copies/ml.[30]

Analysis of intrahepatic HBV DNA and HBV RNA

DNA and RNA extraction from liver biopsies

DNA and RNA were extracted from snap-frozen human liver needle biopsies or liver resection samples as described previously[31] and detailed in Supplementary information following the recommendations of an international study group.[32]

Quantification of tHBV DNA, cccDNA, and 3.5-kb RNA in liver samples by ddPCR

The quantification of the absolute copy numbers of intrahepatic tHBV DNA, cccDNA, and 3.5-kb RNA was performed using the QX100™ Droplet Digital™ PCR System (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions and detailed in Supplementary information.

Statistical analysis

All data were analysed using Prism GraphPad version 8.1.2 (GraphPad Software, San Diego, CA, USA). Data were expressed as median [IQR] for continuous variables and as count (percentage) for categorical variables. Statistical correlations were tested using the Pearson test for normal variables and the Spearman test for other variables. Categorical variables were compared using the Chi-square test or Fischer's exact test, and quantitative variables were compared using Student's *t* test or nonparametric tests (the Mann–Whitney or Kruskall–Wallis test), when appropriate. The significance α threshold was set at 0.05.

Results

Patient characteristics

A total of 54 patients were initially screened at the time of registration on the OLT waiting list between 2012 and 2015: of them, 31 underwent an OLT for CHB and were included in the present prospective study. The median [IQR] age at the time of OLT of patients with HBV was 57.3 [49.4–63.4] years, and 27 (87%) were males. At OLT registration, 22 (71%) patients had an HCC, and five of them had an active HCC at the time of OLT. The median [IQR] delay between HBV diagnosis and OLT was 8.8 [3.0–15.5] years. Before OLT, 30/31 (97%) patients had an antiviral treatment (for a median [IQR] duration of 28.7 [13.2–75.9] months), with most of them receiving entecavir (38%) or tenofovir (38%) (Table 1). Of the 31 patients, three had detectable serum viral load at the time of OLT (median [IQR] value at 175 [131–334] IU/ml) (Table 1). No patient was coinfected with HIV or HCV, and three patients with HDV-positive serology were excluded from the study. In addition, six HBV-negative patients who underwent a transplant were included as technical negative controls: three underwent a transplant for alcohol-related

Table 1. Patients' characteristics at the time of OLT.

Clinical characteristics	HBV group (n = 31)
Age at OLT, median [IQR] (years)	57.3 [49.4–63.4]
Male/female sex ratio, n (%)	27 (87.1%)/4 (12.9%)
BMI, median [IQR] (kg/m^2)	24.0 [22.1–26.8]
MELD score at OLT registration, median [IQR]	11.1 [6.1–21.3]
Child–Pugh score at OLT registration, median [IQR]	B7 [A5–B9]
A, n (%)	13 (41.9%)
B, n (%)	10 (32.3%)
C, n (%)	8 (25.8%)
Waiting time on the waiting list, median [IQR] (days)	162 [90.5–344]
Blood group, n (%)	
O	15 (48.4%)
B	7 (22.6%)
A	7 (22.6%)
AB	2 (6.5%)
Ethnicity, n (%)	
African*	11 (35.5%)
Caucasian	10 (32.3%)
Asian	6 (19.4%)
North African*	2 (6.5%)
Others	2 (6.5%)
HCC (presence)	22 (71%)
Number of nodules, median [IQR]	2.2 [1.0–2.8]
Size of the largest nodule, median [IQR] (mm)	24.2 [15.0–30.5]
Serum AFP level, median [IQR] (μg/L)	5.0 [3.0–12.8]
Patient with active HCC at OLT time	5/22
Patient with HCC treated before OLT	20/22
History of chemoembolisation	12
History of surgical resection	6
History of thermo-ablation	5
History of other treatment	3
Virological status at the time of OLT	
Delay between HBV diagnosis and OLT, median [IQR] (months)	8.8 [3.0–15.5]
HBeAg positive, n (%)	2 (6.5%)
Serum viral load (detectable/undetectable), n (%)	3 (9.7%)/28 (90.3%)
Level of detectable serum viral load, median [IQR] (IU/ml)	175 [131–334]
HBV treatment before OLT, n (%)	
Entecavir	13 (38.2%)
Tenofovir	13 (38.2%)
Lamivudine	3 (8.8%)
Tenofovir + emtricitabine	1 (2.9%)
None	1 (2.9%)
Duration of antiviral treatment before OLT, median [IQR] (months)	28.7 [13.2–75.9]

AFP, alpha-foetoprotein; HCC, hepatocellular carcinoma; MELD, model for end-stage liver disease; OLT, orthotopic liver transplantation.
* North Africa includes Tunisia, Libya, Morocco, Egypt, and Algeria. Africa refers to other countries on the continent, not included in the previous list.

disease and three for chronic hepatitis C (all were negative for HBV markers in the liver and serum; data not shown). All graft donors were anti-hepatitis B core (HBc) negative.

The M12 survival was 100%. All patients received a dual prophylaxis regimen of NUC and HBIG: all patients had i.v. HBIG administration during the anhepatic phase and during the first week after OLT. After the first week, 22/31 (71%) patients were administered HBIG i.v. and 9/31 (29%) s.c. (Table 2). Median [IQR] levels of anti-hepatitis B surface (HBs) antibodies at M3 and M12 were 825.0 [629.5 to >1000.0] mIU/ml and 487.0 [395.3 to >1,000] mIU/ml, respectively. As immunosuppressive regimen, 61% of the patients received tacrolimus and mycophenolate mofetil, around 13% of patients received tacrolimus and everolimus, or tacrolimus alone, and only one patient received cyclosporin alone. Three patients received other drug regimens (Table 2). All patients were negative for serum HBsAg with conventional assays at M3 and M12. Serum HBV DNA was undetectable at M3 and M12 in all patients (Table 2). Four patients underwent a second OLT: two for a primary graft non-function, one for ischaemic cholangiopathy, and one for a 'small for size' syndrome. No retransplantation was performed for HBV recurrence. Median [IQR] delay before the first and second OLT was 26.5 [16.0–83.5] days. Two biopsies at M12 were from these patients who underwent a retransplant.

Table 2. Post-OLT patient characteristics.

Clinical characteristics	HBV group (n = 31)
Type of NUC, n (%)	
Tenofovir	16 (52%)
Entecavir	14 (45%)
Lamivudine	1 (3%)
HBIG after OLT	
HBIG treatment duration, median [IQR] (days)	358 [332–406]
Route of HBIG perfusion after the first week, n (%)	
i.v.	22 (71%)
s.c.	9 (29%)
Immunosuppressive regimen at M12, n (%)	
Tacrolimus and mycophenolate mofetil	19 (61%)
Tacrolimus and everolimus	4 (13%)
Tacrolimus alone	4 (13%)
Cyclosporine alone	1 (3%)
Others	3 (13%)
Virological status at M3 after OLT	
Conventional-HBsAg positive, n (%)	0 (0%)
Anti-HBs-antibodies positive, n (%)	31 (100%)
Anti-HBs antibody level, median [IQR] (mIU/ml)	825 [629.5 to >1,000.0]
Serum HBV DNA detectable, n (%)	0 (0%)
Virological status at M12 after OLT	
Conventional HBsAg positive, n (%)	0 (0%)
Anti-HBs antibodies positive, n (%)	31 (100%)
Anti-HBs antibody level, median [IQR] (mIU/ml)	487 [395.3 to >1,000.0]
Serum HBV DNA detectable, n (%)	0 (0%)
Liver enzymes at M12 after OLT	
ALT, median [IQR] (IU/L)	21.3 [16.0–28.8]
AST, median [IQR] (IU/L)	27.2 [22.0–31.0]
GGT, median [IQR] (IU/L)	30.0 [21.0–64.8]
AP, median [IQR] (IU/L)	82.0 [78.5–100.0]
Bilirubin, median [IQR] (mg/dl)	0.5 [0.4–0.6]

ALT, alanine aminotransferase; AP, alkaline phosphatase; AST, aspartate aminotransferase; HBIG, hepatitis B immunoglobulin; GGT, gamma-glutamyl transferase; M12, 12 months after OLT; M3, 3 months after OLT; NUC, nucleos(t)ide analogue; OLT, orthotopic liver transplantation.

At M12, liver function tests were in the normal range for the majority of patients, and only three had increased gamma-glutamyl transferase in the context of obesity (Table 2).

Quantification of HBV markers in liver samples

At the time of OLT, 27 biopsy samples of liver explant were available from the 31 HBV-positive patients. Among these samples, 26/27 (96%) were positive for tHBV DNA, 21/27 (77%) for cccDNA, and 19/27 (74%) for 3.5-kb HBV RNA. The median [IQR] tHBV DNA, cccDNA, and 3.5-kb HBV RNA levels were 0.077 [0.009–0.19], 0.002 [0.0009–0.007], and 0.012 [0.003–0.05] copies/cell, respectively. The median [IQR] cccDNA transcriptional activity, estimated as the 3.5-kb HBV RNA/cccDNA ratio in the explanted livers, was 8.38 [0.5–36.8] (Fig. 1).

The reperfusion biopsies were collected within a median [IQR] delay between portal vein anastomosis and biopsy of 48 [41–62] min. Of 26 reperfusion biopsies available, 7 (27%) were positive for tHBV DNA, and the median [IQR] concentration was 0.005 [0.00007–0.03] copies/cell (Fig. 1A). At M12, among the 17 liver biopsy samples available, 9 (53%) were positive for tHBV DNA, and the median [IQR] concentration was 0.00045 [0.0002–0.0008] copies/cell (Fig. 1A).

Regarding cccDNA quantification, 11/26 (42%) reperfusion biopsies were positive, and the median [IQR] concentration was 0.001 [0.0001–0.011] copies/cell (Fig. 1B). At M12, 8/17 (47%) biopsy samples were cccDNA positive, and the median [IQR] concentration was 0.0004 [0.0001–0.003] copies/cell (Fig. 1B).

In 2/26 (8%) reperfusion biopsies, 3.5-kb HBV RNA was detected, and the median [IQR] concentration was 0.0011 [0.001–0.016] copies/cell. At M12, 6/17 (35%) biopsy samples were 3.5-kb RNA positive, and the median [IQR] concentration was 0.00006 [0.00003–0.006] copies/cell (Fig. 1C). Given the very low intrahepatic viral load after transplantation, only one sample was positive for both cccDNA and 3.5-kb HBV RNA on the reperfusion biopsy and three samples were positive at M12, and thus, these samples could not be used for the calculation of cccDNA transcriptional activity (Fig. 1D).

Quantification of intrahepatic HBV markers in longitudinal samples

In 14 patients, native liver sample, sequential reperfusion, and M12 biopsies were available. All of them had quantifiable HBV markers, either DNA or RNA, or both, in the native liver. Seven of 14 (50%) patients had quantifiable HBV markers on the reperfusion sampling, and six of them (86%) were also positive at M12. Of the seven patients with HBV-negative reperfusion biopsy, four (57%) presented quantifiable HBV markers at M12. Thus, only three of 14 patients remained negative for HBV markers quantification at both reperfusion and M12 sampling. Overall, 10/14 (71%) patients had HBV-positive M12 liver biopsy, and six of them had already quantifiable levels of HBV markers in their reperfusion liver sample.

Ultrasensitive HBsAg and HBcrAg quantification and cirB-RNA in serum

At the time of inclusion in the study (referred to as baseline), all 30 available samples were positive for serum HBsAg (determined using the Lumipulse assay), and the median [IQR] HBsAg concentration was 572,282 [67,374–1,156,666] mIU/ml. In addition, 22/30 (73%) samples were positive for HBcrAg, and the median [IQR] concentration was 4.2 [3.7–4.9] logU/ml. The serum HBV RNA level could be quantified in 12/30 (40%) samples, and the

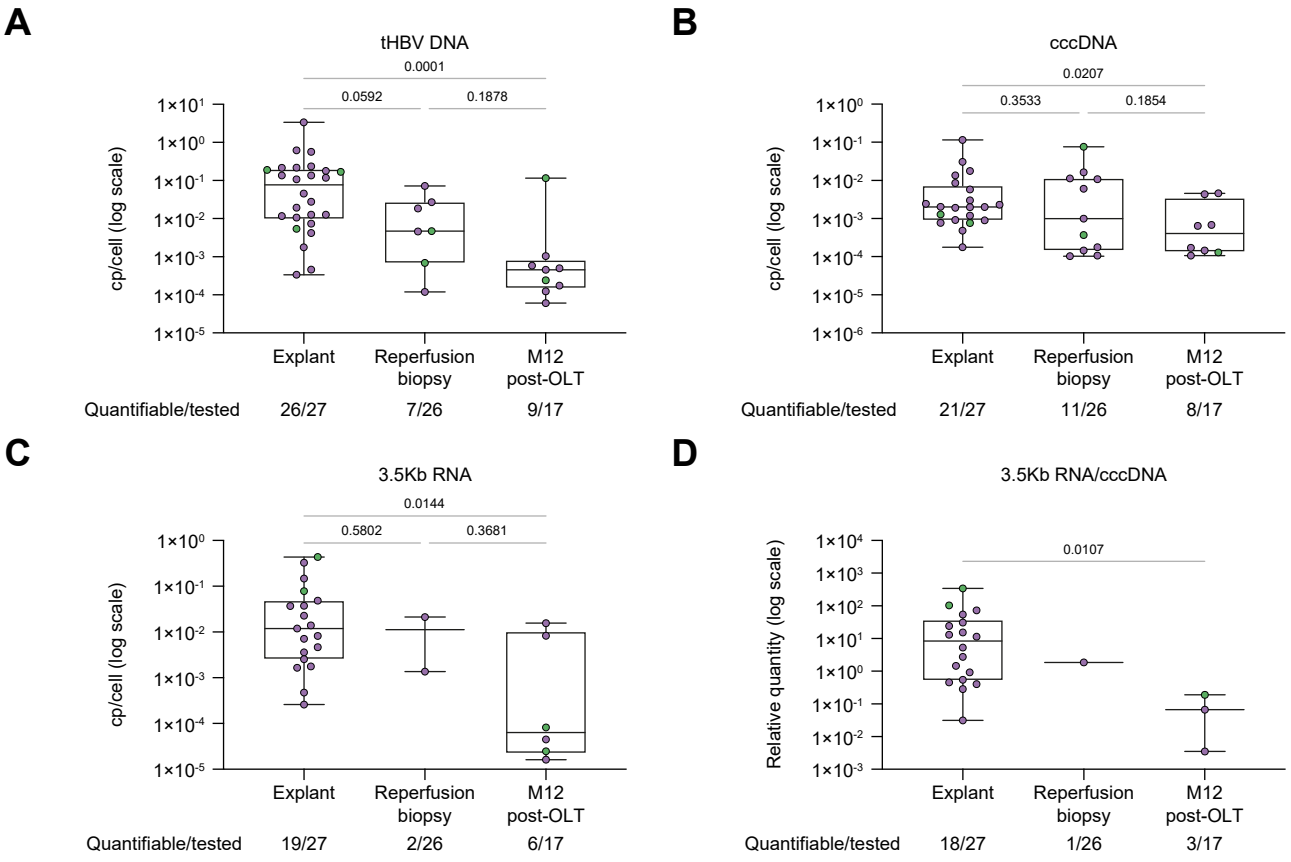

Fig. 1. Evolution of (A) tHBV DNA, (B) cccDNA, (C) 3.5-kb HBV RNA, and (D) cccDNA transcriptional activity on liver explant, on reperfusion biopsy, and at M12. Green dots: patients with detectable serum HBV DNA before OLT. The Mann–Whitney test was used to compare the groups (α threshold = 0.05). cccDNA, covalently closed circular DNA; LLoQ, lower level of quantification; M12, 12 months after OLT; OLT, orthotopic liver transplantation; tHBV, total HBV.

median [IQR] concentration was 143.5 [27.7–498.8] copies/ml (Fig. 2).

Serum HBV markers were then evaluated in samples collected at M3 and M12. At M3, one of 29 (3%) samples were positive for HBsAg (determined using the Lumipulse assay; 14.6 mIU/ml; Fig. 2A). At M12, although all samples were negative, as determined using conventional HBsAg assays, five of 27 (18%) patients had a quantifiable HBsAg, determined using ultrasensitive quantification, with a median [IQR] concentration of 13.9 [11.0–15.1] mIU/ml (Fig. 2A).

At M3, 7/29 (24%) samples were positive for HBcrAg, with six of them having quantifiable values (median [IQR] concentration, 3.2 [3.1–3.3] logU/ml) (Fig. 2B). At M12, 8/27 (30%) samples were positive for HBcrAg; the median [IQR] concentration was 3.2 [3.1–3.2] logU/ml and was determined for the five patients with quantifiable values (Fig. 2B).

HBV RNA was undetectable in all serum samples analysed at M3 and M12 (Fig. 2C).

Correlation between intrahepatic and serum viral markers at baseline

The pre-OLT baseline serum HBcrAg level was significantly correlated with the tHBV DNA (r = 0.74; p <0.0001), cccDNA (r = 0.61; p = 0.0004), and 3.5-kb HBV RNA (r = 0.84; p <0.0001) levels in the native liver. Pre-OLT ultrasensitive serum HBsAg concentration was correlated with the tHBV DNA (r = 0.62; p = 0.0008) and 3.5-kb HBV RNA (r = 0.55; p = 0.0032) levels, but not with the intrahepatic cccDNA level (r = 0.20, p = 0.21) in the native liver. Before OLT, the cirB-RNA level was not correlated with the cccDNA level (r = 0.3090; p = 0.055) and tHBV DNA (r = 0.118; p = 0.263) in the native liver. cirB-RNA was correlated with intrahepatic 3.5-kB HBV RNA (r = 0.714; p = 0.002) and with transcriptional activity of cccDNA (r = 0.640; p = 0.029) in the native liver.

Association between intrahepatic and serum viral markers at M12

As stated above, 5/27 patients had positive and quantifiable serum HBsAg levels using the ultrasensitive assay at M12 while being undetectable using conventional methods (Fig. 3A). M12 biopsies were available for three of these five patients; HBV DNA could be detected in all of them (3/3 for cccDNA and 2/3 for tHBV DNA), and one was also positive for 3.5-kb HBV RNA (Fig. 3A). The corresponding reperfusion biopsy samples were all negative for intrahepatic viral markers, except one, which had quantifiable HBV DNA (both tHBV DNA and cccDNA) (Fig. 3A).

HBcrAg was detectable in eight of 27 samples at M12, with quantifiable values (≥3 logU/ml) in five of them. Six of these eight patients had a M12 biopsy available, and 30% of them were positive for cccDNA detection (Fig. 3B). Six reperfusion biopsy samples of these eight patients were available: all were negative except one, which had quantifiable levels of both cccDNA and tHBV DNA.

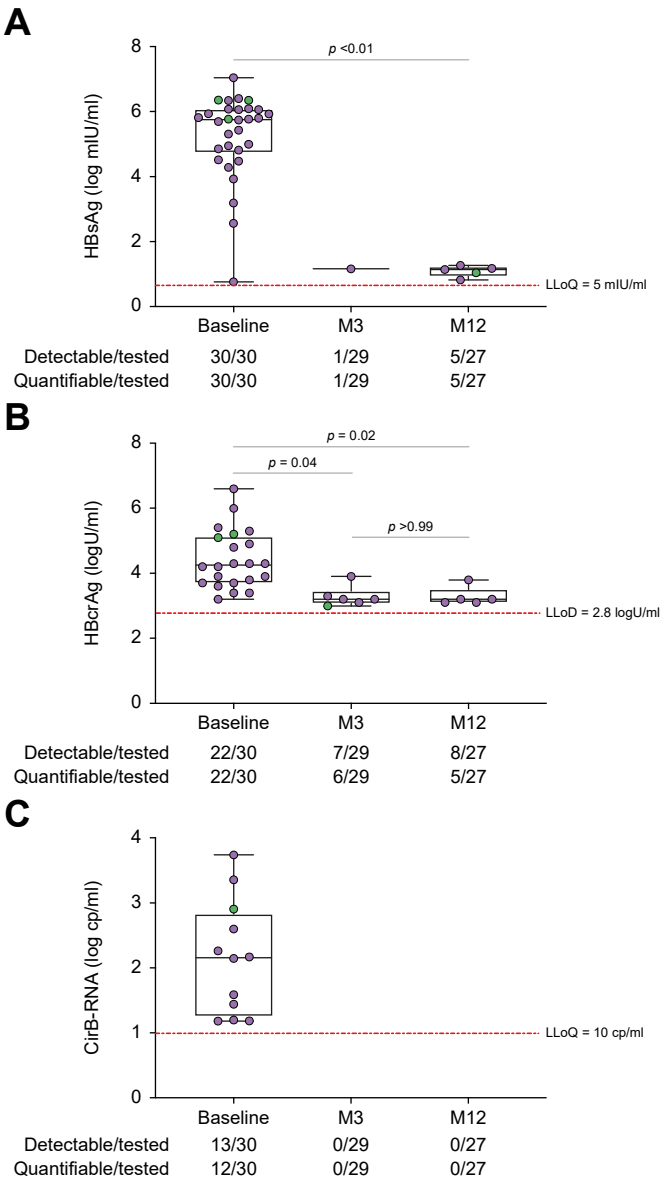

Fig. 2. Evolution of serum (A) HBsAg, (B) HBcrAg, and (C) HBV RNA quantification before OLT and at M3 and M12. Green dots: patients with detectable serum HBV DNA before OLT. The Mann–Whitney test was used to compare the groups (α threshold = 0.05). cirB-RNA, circulating HBV RNA; LLoQ, lower level of quantification; M12, 12 months after OLT; M3, 3 months after OLT; OLT, orthotopic liver transplantation.

At M12, one patient (one of 32) had quantifiable values for both ultrasensitive HBsAg and HBcrAg. The analysis of the reperfusion biopsy showed undetectable levels of HBV markers, whereas only cccDNA was detectable in the M12 biopsy.

We did not find any difference between the duration of antiviral therapy before OLT and HBV recurrence infection on the graft at M12, defined by tHBV DNA ($p = 0.26$), cccDNA ($p = 0.38$), 3.5-kb HBV RNA ($p = 0.25$), HBsAg ($p = 0.24$), or HBcrAg ($p = 0.25$) levels. There was no correlation between HBV recurrence on the graft at M12 and anti-HBs antibody levels at M3 and M12, nor was there correlation with the HBIG route injection type (s.c. or i.v.), the presence of active HCC at OLT, or the type of immunosuppressive regimen. The two patients who underwent a second liver transplant had undetectable quantitative HBsAg and HBcrAg in the serum, whereas one M12 biopsy had quantifiable intrahepatic HBV DNA and RNA.

Finally, among the 17 patients having a matched biopsy and serum sample at M12, only one had undetectable HBV markers in both the liver and serum compartments. These 17 patients did not differ from the 14 others (with no matched biopsy and serum sample at M12) on clinical characteristics at the time of OLT and on virological parameters on explant.

Discussion

The present study, based on the analysis of both cross-sectional and sequential serum and liver samples, showed that despite the undetectability of the conventional HBV markers in the serum, HBV recurrence can occur very early after OLT in a majority of patients receiving an anti-HBc-negative graft.

During long-term NUC administration, intrahepatic and serum HBV DNA levels are decreased to very low levels, resulting in their undetectability with classical quantitative PCR (qPCR) techniques in most cases.[26,33,34] Nevertheless, qPCR-negative patients have been shown to experience viral relapse after treatment withdrawal, thus suggesting an undetected residual cccDNA pool in their liver.[35,36] At the time of OLT, in the present study, patients were receiving NUC for a median duration of 28.7 months, and serum HBV DNA was undetectable for most of them. However, using a ddPCR assay, we were able to detect HBV nucleic acids in almost all available liver explants. These data are consistent with those of immunocompetent patients with CHB under long-term NUC therapy viral suppression or those of patients with occult HBV infection.[26,27,37,38] The intrahepatic cccDNA levels in the liver explants were slightly lower than those reported in patients with CHB who did not undergo a transplant and are under long-term NUC therapy.[26]

Thanks to the access to sequential liver samples after OLT in a subset of patients, we could investigate the time course of HBV recurrence on the graft using ddPCR assays. Interestingly, our results showed a very early recurrence of HBV in the grafted liver in half of reperfusion biopsy samples, despite i.v. HBIG perfusion during the operative period. Several hypotheses can be put forward to explain this early HBV recurrence. First, residual viral particles present in the blood because of incomplete viral suppression, or release from the liver during hepatectomy, or viral particles present in extrahepatic reservoirs during the anhepatic phase, may infect the new liver.[39–41] Indeed, it has been shown using experimental models that residual low viraemia levels in a context of NUC therapy can be infectious in liver-humanised mice[42] and that NUCs do not prevent the *de novo* formation of cccDNA in infected hepatocytes in cell culture or *in vivo*.[17,18] Assuming that HBV could circulate just below the lower limit of detection of PCR assays in the NUC-suppressed patients awaiting OLT, up to 10^5 HBV copies could be present in the blood circulation at the time of surgery and may be thus available to infect the estimated 10^{11} hepatocytes of the graft. This remaining viral burden may not be fully neutralised by the infusion of HBIG during the anhepatic phase. It has also been shown that HBV mutants may escape both HBIG and NUC and infect the liver graft despite prophylaxis;[43,44] however, it is unlikely that this phenomenon contributed to the graft infection, as serum HBV DNA remained nonquantifiable by qPCR in all patients. Lastly, recent

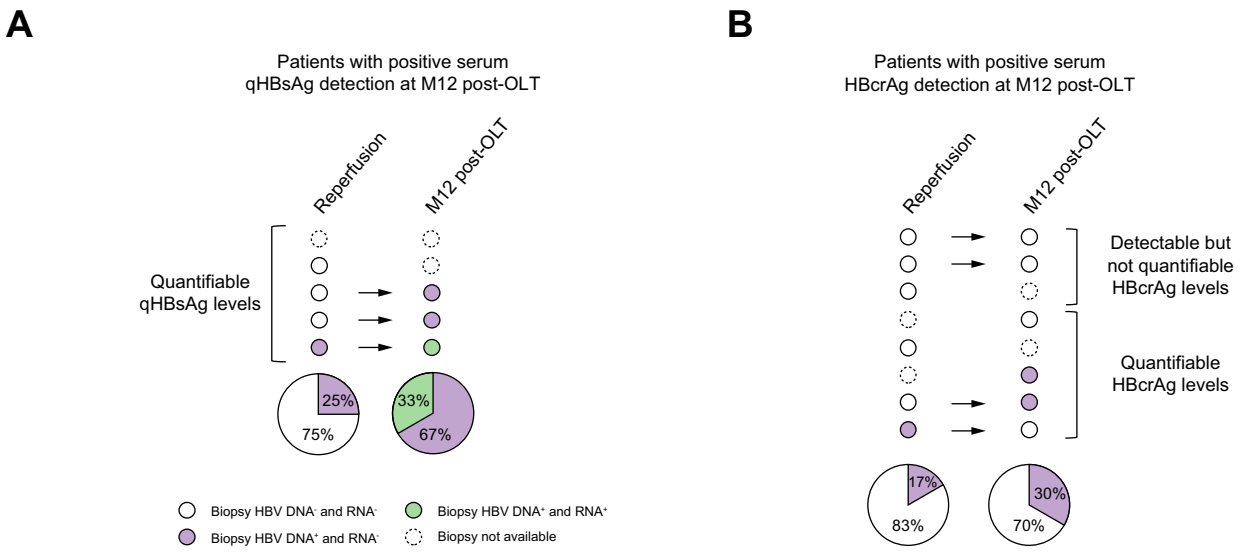

Fig. 3. Representation of ultrasensitive (A) HBsAg- or (B) HBcrAg-positive patients according to HBV intrahepatic markers on reperfusion biopsy and at M12. Each circle represents a liver sample at reperfusion or M12. White circles: negative quantification for both HBV DNA and RNA; purple circles: positive quantification for HBV DNA and negative for HBV RNA; and green circles: positive quantification for both HBV DNA and RNA. Circles with shaded profile: biopsy not available. To be noted: HBV DNA indicates either tHBV DNA or cccDNA quantification. cccDNA, covalently closed circular DNA; M12, 12 months after OLT; OLT, orthotopic liver transplantation; qHBsAg, quantitative HBsAg; tHBV, total HBV.

in vitro data indicated that formation of cccDNA in the nucleus of infected hepatocytes occurs rapidly after viral inoculation,[45,46] being detectable by qPCR between 30 min and 2 h post infection,[46] thus accounting for the possibility of a very early infection of the graft that could be already detectable at the time of the reperfusion biopsy.

At M12, cccDNA could be detected at very low levels in half of the available samples, and the associated cccDNA transcription rates were very low. Overall, at least tHBV DNA or cccDNA could be detected at M12 in the majority of samples, suggesting a significant rate of infection of the graft, which remained under control thanks to the antiviral prophylaxis protocol. Altogether, the results are consistent with the effectiveness of the HBIG and NUC prophylaxis in maintaining a strong antiviral pressure and suggest that very few hepatocytes might harbour cccDNA. Such a low viral burden in the liver graft would certainly not be detected by conventional immunostaining assays. The development of new technologies for single-cell genomic and proteomic analysis may allow further investigations to address this question.[47]

When analysing novel serum viral biomarkers, we found that, at M12, five patients were positive for HBsAg quantified using an ultrasensitive method and eight were positive for HBcrAg, although all patients tested negative for both serum HBsAg and HBV DNA with conventional assays. In these patients, the HBsAg concentrations remained very low, and the anti-HBs antibody concentration was high at M3 and M12. A possible explanation for this observation could be the presence of immune complexes masking HBsAg or the presence of HBs mutants escaping HBs antibody recognition in the conventional assay.[43,44,48] Because patients received NUC therapy, serum HBV DNA remained undetectable by qPCR. The long-term consequences of HBsAg seropositivity after OLT are currently unknown. Ultrasensitive serum HBsAg quantification correlated with tHBV DNA and 3.5-Kb HBV RNA in the explanted liver, but not with cccDNA. This was consistent with the findings in immunocompetent patients with CHB.[49] The significant number of patients with positive detection of serum HBcrAg at M12 was in contrast with the very low detection of intrahepatic 3.5-kb RNAs, the only viral transcript species able to generate both HBeAg and HBcAg. We cannot rule out that this could be as a result of limitations in sensitivity of intrahepatic RNA detection or in the specificity of the HBcrAg assay.

After OLT, in the few patients with a positive HBcrAg or HBsAg quantification, no obvious correlation was found with the levels of intrahepatic viral markers. This is in contrast to the findings by Matsuzaki *et al.*,[24] who found a positive correlation between serum HBcrAg and intrahepatic cccDNA levels determined by a less sensitive qPCR analysis after OLT; however, detailed information on the prophylaxis regimen was not reported.[24]

One limitation of our prospective cohort study is that it was not designed as a comparative clinical trial of long-term *vs.* short-term HBIG. The early HBV infection of the liver graft tissue and the fact that HBsAg could be detected using an ultrasensitive method in the serum of some patients at M12 underline the importance of an optimal patient compliance to the antiviral prophylaxis to prevent viral rebound, regardless of the duration of HBIG administration.

In conclusion, this study investigating viral markers in both the liver and serum compartments on sequential samples showed that the recurrence of HBV infection can occur very early after the transplantation despite standard HBIG and NUC prophylaxis. Thus, our data strongly emphasise the importance of optimal patient compliance to antiviral therapy and raise the question of the dosage and duration of HBIG prophylactic regimen. Randomised studies would be warranted to specifically address the impact of HBIG prophylactic therapy *vs.* HBIG-free regimens on early tissue reinfection and its correlation with clinical outcome.

Abbreviations

AFP, alpha-foetoprotein; cccDNA, covalently closed circular DNA; CHB, chronic hepatitis B; cirB-RNA, circulating HBV RNA; ddPCR, Droplet Digital PCR; HBc, hepatitis B core; HBcrAg, hepatitis B core-related antigen; HBIG, hepatitis B immunoglobulin; HBs, hepatitis B surface; HCC, hepatocellular carcinoma; M12, 12 months after OLT; M3, 3 months after OLT; NUC, nucleos(t)ide analogue; OLT, orthotopic liver transplantation; tHBV, total HBV.

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Conflicts of interest

FV, FL, SR, BR, MJY, VL, ST, ML, SSA, CS, and BT declare no conflicts of interest. FZ received consulting fees from Aligos, Antios, Assembly, Gilead, and GSK, and research funding to INSERM from Asssembly, Beam, and Janssen. DS received consulting fees from Biotest, Go Liver, Gilead Sciences, and Seabelife. RA received consulting fees from Gilead and research funding to CHU of Nice, France, from: Novartis. GPP received fees from Gilead. AH and MH are employees and stakeholders of Roche Molecular Diagnostics.

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

Authors' contributions

Concept and design: FZ, BT, FV, SSA. Methodology and investigation: FV, BT, FB, CS, AH. Formal analysis and visualisation: FV, BT. Data curation: ST. Writing – original draft: FV, BT, FZ. Writing – review and editing: BT, FZ, ML, FL, DS, VL, MH, SR, BR, JYM, VL, ML, FZ, DS, RA, GPP. Supervision and funding acquisition: FZ. Resources: MH, FL, SR, BR, JYM, VL, ML, FZ, DS, RA, GPP. Had access to the study data and reviewed and approved the final version of the manuscript: all authors.

Data availability statement

De-identified individual participant data that underlie the reported results will be made available upon request 3 months after publication for a period of 5 years after the publication date.

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Supplementary data

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Author names in bold designate shared co-first authorship.

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