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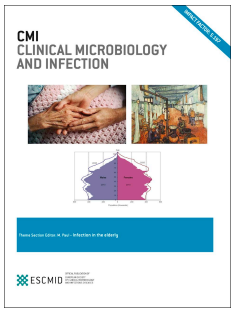

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Original paper

Persistent risk of HBV reactivation despite extensive lamivudine prophylaxis in haematopoietic stem cell transplant recipients who are anti-HBc-positive or HBV-negative recipients with an anti-HBc-positive donor

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Abstract

Objectives - The overall rate of HBV reactivation was evaluated in a population of 373 haematological stem cell transplant (HSCT) patients treated with lamivudine (LMV) if they were anti-HBc-positive/HBV-DNA-negative recipients or if they were HBV-negative recipients with an anti-HBc-positive donor.

Methods - The incidence of HBV reactivation was calculated in two groups of autologous (auto) or allogeneic (allo) HSCT patients who were stratified according to their HBV serostatus. The former group included 57 cases: 10 auto-HSCT and 27 allo-HSCT anti-HBc-positive recipients, 2 auto-HSCT and 3 allo-HSCT inactive carriers and 15 allo-HSCT recipients with an anti-HBc-positive donor. Forty-seven (82.4%) patients in this group received LMV prophylaxis (the median [interquartile range-IQR] of LMV treatment was 30 [20-38] months). The second group consisted of 320 anti-HBc-negative auto-HSCT and allo-HSCT recipients with anti-HBc-negative donors. None of these patients received any prophylaxis.

Results - Two patients in the first group and 2 in the second group experienced reactivation of HBV infection, with an incidence of 3.5% [95% C.I: 0.4%-12.1%] and 0.6% [95% C.I.: 0.1%-2.2%], respectively. Only 1 out of 4 reactivated patients was LMV-treated. The cumulative probability of HBV reactivation at 6 years from HSCT was 15.8% [95%C.I.: 15.2%-16.4%]. Three of four viral isolates obtained from the HBV-reactivated patients harboured mutations in the immune-active HBsAg-region.

Conclusions - In a HSCT population carefully evaluated for HBV prophylaxis, a risk of HBV reactivation persisted in the group of patients who were not LMV-treated. Only one LMV-treated patient experienced reactivation of HBV with a resistant HBV isolate.

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Introduction

HBV infection causes 40% of fulminant hepatitis cases, and 6.8% of these hepatitis cases occur in immunocompromised patients during HBV reactivation (1). A proportion of HBV-reactivated patients may recover spontaneously, but the mortality related to HBV reactivation in immunocompromised subjects ranges from 5% to 40% (2,3). Haematological patients receiving high-dose chemotherapy, rituximab treatment and/or undergoing haematopoietic stem cell transplantation (HSCT) are at major risk for HBV reactivation (1- 6).

Prophylactic antiviral therapy significantly reduces the frequency of reactivation in hepatitis B antigen S (HBsAg) carriers (7), and several international guidelines recommend screening and prophylaxis for HBV for all patients undergoing chronic immunosuppressive therapy (8, 9). However, pre-chemotherapy HBV screening occurs less frequently than expected, and there is a documented overall low rate of prophylactic use of antiviral therapy (10-15).

HSCT recipients are at a high risk of HBV reactivation because of the underlying disease, the intense chemotherapy that is initially administered to treat the malignancy and the subsequent chemo/radiotherapy that is administered as a pre-transplant conditioning regimen. The HBV reactivation rates in the HSCT population vary from 6% to 50% (16-22), and this variability is related to several factors, such as the patient's regional HBV prevalence, age, gender, donor/recipient HBV serology, haematological malignancy history and treatment, the type of HSCT, and the presence and severity of graft-versus-host disease (GvHD) (18, 21-24).

Current international guidelines on HBV prophylaxis for HSCT patients are limited and generically include HSCT in the general chapter of immunosuppression (8, 25, 26). However, the high prevalence of occult HBV infection (OBI) (defined as the presence of HBV-DNA in the liver with or without HBV DNA in the serum of individuals who test negative for HBsAg) (27) in haematological patients, the possibility that CD34+ cells are carriers of HBV, and the relevance of donor HBV conditions (17, 19, 28-30) mean that HSCT patients require specific HBV screening and prophylaxis/treatment measures.

The recent 5th European Conference on Infections in Leukaemia (31) suggested that HBV prophylaxis should be used in HBsAg-positive or antibody versus HBc-antigen (anti-HBc)-positive HSCT recipients as well as HBV-negative recipients of transplants from anti-HBc-positive donors.

We considered the use of lamivudine (LMV) prophylaxis for anti-HBc-positive transplant recipients and recipients of transplants from an anti-HBc-positive donor from 2007 onwards in the Stem Cell Transplant Unit of Tor Vergata University, Rome.

This study evaluated the overall rate of HBV reactivation in our centre and examined the characteristics of viral isolates that characterize this phenomenon.

Materials and Methods

Study population and HBV testing

A total of 373 adult patients who were scheduled for an autologous (auto-HSCT) or allogeneic HSCT (allo-HSCT) at the University Hospital of Tor Vergata, Italy between January 2008 and December 2013 were screened for HBV serological markers (HBsAg anti-HBs, anti-HBc, HBe antigen [HBeAg] and HBe antibodies [anti-HBe]) and serum HBV-DNA levels and reviewed for HBV reactivation occurrence.

The patients were identified as having a resolved HBV infection if they were anti-HBc-positive (+/- anti-HBs-positive), HBsAg-negative and serum HBV-DNA-negative at pre-transplant evaluations. Patients who were HBsAg-positive, HBeAg-negative and anti-HBe-positive with serum HBV-DNA <2,000 IU/mL and persistently normal transaminase levels were defined as inactive carriers. Patients who were anti-HBs-positive and anti-HBc-negative were considered vaccinated.

All HSC donors were tested for HBsAg, anti-HBc, anti-HBs and HBV-DNA prior to donation in the setting of allogeneic transplantation.

HBV reactivation was defined as the reappearance of serum HBV-DNA or a marked increase in the serum HBV-DNA (>1 log IU/mL) from the baseline level during or after the administration of immunosuppressive therapy according to criteria established by the American Association for the Study of Liver Disease (32).

The following information was recorded for each patient: age at the time of transplantation, sex, transplant conditioning regimen (myeloablative conditioning [MAC] or reduced

intensity conditioning [RIC]), stem cell source, grade of acute GvHD (aGvHD) and grade of chronic GvHD (cGvHD).

The following data were evaluated and monitored over time in the group of patients who developed HBV reactivation: alanine aminotransferase (ALT), aspartate transaminase (AST), serum HBV-DNA levels, HBsAg, anti-HBs and HBV genotype.

The trends in the results of the liver function tests (ALT, AST, bilirubin, alkaline phosphatase, gamma-glutamyl transpeptidase, prothrombin time and fibrinogen) were periodically evaluated in all HSCT patients during LMV prophylaxis. The study did not provide a standardized protocol for monitoring HBsAg and HBV-DNA levels during LMV prophylaxis; however, serological HBV markers and HBV-DNA levels were evaluated in all patients before LMV treatment was suspended (median number of samples [IQR] available for patients of 2 [1-3]).

All of the patients' personal information was treated in a confidential manner, and all clinical data were collected anonymously to respect the patients' privacy. The study was approved by the Tor Vergata University Ethical Committee.

Allo-HSCT conditioning

Uniform conditioning (called TBF) was used for all transplanted patients (33). The MAC version of this regimen consisted of a combination of thiotepa (5 mg/kg over 2 days), intravenous busulfan (3.2 mg/hg three-hour infusion over 3 days) and fludarabine (50 mg/m^2 over 3 days). Patients with a Sorror index >2 or aged >55 years received the RIC version of TBF by deleting one dose of thiotepa and one dose of busulfan. Acute and chronic GvHD were graded using standard criteria (34).

HBV prophylaxis

LMV treatment (100 mg/day) was administered before transplantation to all patients who presented serological signs of a previous HBV infection (inactive carrier or resolved infection) or recipients from an anti-HBc+/-anti-HBs-positive donor. The duration of prophylaxis was established based on the expected time for immunological recovery. Auto-HSCT patients interrupted their LMV treatment 12-18 months after the discontinuation of all immunosuppressive drugs. Allo-HSCT patients were generally treated for a longer period of time (over two years), and LMV interruption corresponded with the achievement of a CD4+ lymphocyte count greater than or equal to 400 cells/mm[c].

Laboratory testing for the diagnosis of HBV infection

The levels of serological HBV markers were measured using immune-enzymatic assays (Roche/Cobas® Diagnostics, Rotkreuz, Switzerland). Plasma HBV-DNA was identified in the study period (2008-2013) using real-time polymerase chain reaction (lower limit of quantification: 20 IU/mL) (Roche/Cobas® Ampliprep/Cobas® Taqman, Rotkreuz, Switzerland).

Population-based sequencing of HBV reverse transcriptase and HBsAg

Population-based sequencing of HBV reverse transcriptase (RT) and HBsAg was performed on plasma samples at the time of HBV reactivation. Population-based sequencing was used to define the HBV genotype and detect drug-resistance mutations in RT and immune escape mutations in HBsAg.

HBV-DNA was extracted using a commercially available kit (QIAmp DNA blood mini-kit, Qiagen Inc., USA) and amplified with Amplitaq-Gold polymerase using the following primer pairs: F1-5'GGTCACCATATTCTTGGGAA and R1-5'GTGGGGGTTGCGTCAGCAAA. The following polymerase chain reaction (PCR) conditions were used: one cycle at 93 ℃ for 12 min; 40 cycles of 94 ℃ for 50 s, 57 ℃ for 50 s, a nd 72 ℃ for 1 min and 30 s; and a final

cycle at 72 ℃ for 10 min. Two additional hemineste d PCR runs were performed for samples with low serum HBV-DNA levels starting from the same first amplicon: heminested_1 used primer pairs F1-5'GGTCACCATATTCTTGGGAA and R2-GAGGACAAACGGGCAACATACCTT, and heminested_2 used F2-GTTGACAAGAATCCTCACAATA and R1-5'GTGGGGGTTGCGTCAGCAAA. The following heminested PCR conditions were used: one cycle at 93 ℃ for 12 min; 35 cycles of 94 ℃ for 50 s, 56 ℃ for 50 s, 72 ℃ for 1 min; and a final cycle at 72 ℃ for 10 min. The PCR products were purified and sequenced using eight different overlapping sequence-specific primers, a BigDye terminator v.3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) and an automated sequencer (Genetic Analyzer 3130XL). The sequences were analysed using SeqScape-v.2.5 software. The quality endpoint for each individual gene was ensured by coverage of the RT gene sequence by at least two segments. Sequences with a mixture of wild-type and mutant residues at single positions were considered to possess mutant(s) at that position.

The RT sequences were also used to analyse the HBsAg region due to the overlap between the RT and HBsAg genes. Mutations were defined as amino acid variations from the HBV reference sequence. The list of drug-resistance mutations in RT and immune escape mutations in HBsAg was retrieved from the following website: http://hbv.bioinf.mpi-inf.mpg.de/index.php updated on October 2013.

Statistical analysis

The data are expressed as medians (interquartile range [IQR]) for quantitative variables (discrete and continuous) and percentages (in some cases with 95% confidence interval [C.I.]) for qualitative variables. The cumulative incidence of HBV reactivation is reported as the cumulative probability (95% C.I.).

Survival analysis was performed using the cumulative incidence competing risk (CICR) method, which is based on the so-called cumulative incidence function, to estimate the time and cumulative probability of achieving HBV reactivation by accounting for competing risk events represented by transplant-related mortality or death due to the recurrence of haematological disease. This analysis takes into account that HBV reactivation and transplant-related mortality or death due to the recurrence of haematological disease can act as two competing and mutually exclusive events. This analysis allows us to separately estimate the probability of the occurrence of these two events. The time and cumulative probability of achieving HBV reactivation and transplant-related mortality or death due to the recurrence of haematological disease was evaluated starting from the date of transplantation. All data were analysed using the statistical open source environment R (version 3.2.3) and the SPSS statistical software package (version 12; SPSS, Inc., Chicago, IL, USA).

Results

Characteristics of the HSCT patients

Table 1 shows the characteristics of the study population. The patients had a median (IQR) age of 47 (36-55) years, and 52.3% were male. The most represented pathologies were acute myeloid leukaemia (34%), non-Hodgkin's lymphoma (21.2%), multiple myeloma (15.6%), and acute lymphoid leukaemia (12.6%). One hundred seventy-nine (70%) of the 256 allo-HSCT patients received a MAC conditioning regimen. Acute GvHD with a grade >2 developed in 50 patients, and extensive cGvHD occurred in 31 (12%) evaluable patients.

HBV serological profiles in HSCT recipients and donors

A total of 117 and 256 of the 373 enrolled patients underwent auto-HSCT and allo-HSCT, respectively (Table 2). Thirty-seven recipients (9.9%) (10 auto-HSCT and 27 allo-HSCT) were positive for at least anti-HBc: 31 (83.7%) recipients were positive for anti-HBc and anti-HBs, and 6 (16.2%) recipients were only anti-HBc-positive (data not shown). Five patients (1.3%) (2 auto-HSCT and 3 allo-HSCT) were inactive carriers. A total of 133 (35.6%) (23 auto-HSCT and 110 allo-HSCT) of the remaining patients who were anti-HBs-positive were considered previously vaccinated, and 198 (53%) patients (82 auto-HSCT and 116 allo-HSCT) were negative for all HBV markers. With the exception of the inactive carriers, all anti-HBc- and/or anti-HBs-positive patients and donors were HBV-DNA-negative at screening.

Twenty-seven of the 37 (72.9%) patients who were positive for at least anti-HBc underwent allo-HSCT. Ten (37%) of these patients received HSCs from an anti-HBc+/-anti-HBs-positive donor. Seven (6%) of the 116 allo-HSCT recipients were negative for all

HBV serological markers, and 8 (7.2%) of the 110 allo-HSCT recipients with isolated anti-HBs positivity received bone marrow from an anti-HBc+/-anti-HBs-positive donor (Table 2).

Incidence of HBV reactivation based on the presence or absence of HBV markers at screening

The incidence of HBV reactivation was calculated in two groups of HSCT patients who were stratified according to their HBV serostatus at the time of pre-transplant screening. The former group included 57 cases: 37 anti-HBc+/-anti-HBs positive recipients (10 auto-HSCT and 27 allo-HSCT), 5 HBV inactive carriers (2 auto-HSCT and 3 allo-HSCT) and 15 allo-HSCT HBV-negative or anti-HBs-positive recipients with an anti-HBc-positive donor. Forty-seven (82.4%) patients in this group received LMV prophylaxis. The median duration [interquartile range: IQR] of LMV prophylaxis was 30 [20-38] months.

The second group consisted of 320 anti-HBc-negative or anti-HBs-positive (vaccinated) subjects or recipients from HBV-negative or anti-HBs-positive (vaccinated) donors (none of them received any prophylaxis) (Table 3).

The median [IQR] length of follow-up was 2 (1-3) years. The maximum length of follow-up was 6 years.

Two patients in the former group (N=57 patients) exhibited reactivated HBV infection, with an incidence of HBV reactivation of 3.5% [95% C.I.: 0.4%-12.1%] (2/57) (Table 3). One patient was positive for anti-HBc and anti-HBs at the time of screening, and this patient developed HBV reactivation after 41 months of LMV prophylaxis. The other patient was negative for all HBV markers at the time of screening and received HSCs from an anti-HBc-positive donor. This patient escaped prophylaxis assessment and did not receive

LMV. He was the only HBV-negative recipient of a transplant from an anti-HBc-positive donor who exhibited a reactivated HBV infection (14.3% [1/7]).

Two of the 320 patients in the second group exhibited a reactivated HBV infection, with an incidence of HBV reactivation of 0.6% [95% C.I.: 0.1%- 2.2%] (2/320) (Table 3). Notably, these 2 patients were negative for all HBV serological markers at the time of HBV screening. However, an in-depth retrospective analysis of their HBV serological profiles before HSCT screening highlighted that positivity for anti-HBc preceded and was followed by negative HBV serology of the donor in one case. The only anti-HBc-positive value was considered nonspecific at that time.

Finally, the cumulative probability of HBV reactivation was evaluated in the group of 59 patients at 6 years from HSCT, accounting for a competing risk event represented by transplant-related mortality and death due to haematological relapse. In this analysis, HBV reactivation and transplant-related mortality or death due to haematological relapse were considered to act as two competing and mutually exclusive events. The cumulative probability of death due to transplant-related mortality or haematological relapse was 45.5% [CI: 43.5%-47.6%]. Despite this competing risk, the cumulative probability of HBV reactivation was 15.8% [CI: 15.2%-16.4%] (Fig. 1)**.**

Description of the 4 HBV-reactivated patients

Patient 1

Patient 1 was a 62-year-old Italian male. Multiple myeloma was diagnosed in 2010. He underwent auto-HSCT. He was positive for anti-HBc and anti-HBs at the time of serological screening and negative for HBsAg, HBeAg, anti-HBe and HBV-DNA. LMV

prophylaxis was initiated. He underwent HLA identical sibling transplantation from an anti-HBc- and anti-HBs-positive donor in 2011. He received RIC conditioning and was treated for chronic GvHD with low-dose cyclosporine. He experienced HBV reactivation during LMV prophylaxis, 41 months from the first HSCT, during cyclosporine treatment for GvHD. He was also treated with plasmapheresis (last treatment in September 2015) for cGVHD (Table 4).

He had an AST level of 29 U/L, an ALT level of 31 U/L, and a log serum HBV-DNA level of 5.97 IU/mL at the time of HBV reactivation. Therefore, he was HBeAg- and anti-HBc-positive. He had an elevated HBsAg titre (>52.000 IU/mL), and he had lost anti-HBs. HBV RT/HBsAg sequencing detected HBV genotype D1 and the presence of the LMV-resistance mutation L80I (Table 4). Antiviral therapy with tenofovir was initiated. On the 9th month of tenofovir treatment, his HBV-DNA rose to 1000 UI/mL. Despite previous viral drug mutation study, sequencing analysis showed a wild-type virus, and thus entecavir (1 mg/die) was added to tenofovir. The last known HBV-DNA value was 300 UI/mL (November 2015).

Patient 2

Patient 2 was a 63-year-old Italian male. Acute myeloid leukaemia was diagnosed in 2008. He was treated with standard chemotherapy, and he underwent HLA-identical sibling transplantation in July 2008 after RIC conditioning. He was negative for all HBV serological markers and serum HBV-DNA at pre-transplant screening, and he received bone marrow from an isolated anti-HBc-positive donor, but he did not undergo prophylaxis assessment and did not receive lamivudine. He experienced HBV reactivation 30 months later, while receiving steroid prophylaxis treatment (Table 4).

He had an AST level of 2,159 U/L and an ALT level of 2,570 U/L at the time of HBV reactivation, and the log serum HBV-DNA level was 3.24 IU/mL. He was positive for HBeAg and anti-HBc. Notably, the patient was HBsAg-negative and anti-HBs-positive despite HBV reactivation. HBV RT/HBsAg sequencing detected HBV genotype D with several immune-escape mutations in HBsAg (V96 A/V, M103I, T123N, C124Y, T126I, and G145 K/R) and some RT mutations that were potentially associated with drug resistance (A181S and V214A) (Table 4). The T123N HBsAg mutation introduces a new N-linked glycosylation site in HBsAg, which drastically hampers the detection of HBsAg (35). This mutation can explain this patient's negativity to HBsAg.

He died two months later from haematological relapse. His last data suggest a partial reduction of the HBV DNA level to 383 UI/mL but the presence of persistent hypertransaminasemia.

Patient 3

Patient 3 was a 51-year-old Italian female. Multiple myeloma disease was diagnosed in 2010. She underwent auto-HSCT in 2011 and had a relapse of haematological disease in 2013. She underwent MAC conditioning and received an HLA-identical sibling transplant from a donor who was negative for all HBV markers. She experienced GvHD and was treated with a high dose of steroids. HBV serology was negative for all HBV serological markers, and serum HBV-DNA was negative at pre-transplant screening. Therefore, she did not receive any LMV prophylaxis. HBV reactivation occurred 12 months after the first HSCT (Table 4).

She had an AST level of 203 U/L and an ALT level of 361 U/L at the time of HBV reactivation, and the log serum HBV-DNA level was 5.08 IU/mL. Serological markers for HBV were anti-HBc-, HBeAg- and HBsAg-positive (4.029 IU/ml), and anti-HBe and anti-

HBs were negative. She was treated with entecavir, and she obtained a virological response with an HBV DNA level < 2000 UI/mL and transaminase normalization. She died 8 months later from relapse of the haematological disease. HBV RT/HBsAg sequencing detected HBV genotype D3 with an immune-escape mutation in HBsAg (D144E) (Table 4).

Notably, a retrospective analysis of the HBV serological markers of the patient highlighted a weak positivity to anti-HBc in the past.

Patient 4

Patient 4 was a 52-year-old Italian female. Non-Hodgkin's lymphoma was diagnosed in 2010. She was treated with a rituximab-containing regimen and underwent an auto-HSCT. She was negative for all HBV serological markers and serum HBV-DNA at pre-transplant screening, and she did not receive any LMV prophylaxis. She experienced HBV reactivation 52 months later (Table 4).

She had an AST level of 31 U/L and an ALT level of 61 U/L at the time of HBV reactivation, and the log serum HBV-DNA level was 8.94 IU/mL. She had an elevated HBsAg titre (>52.000 IU/mL), and she was HBeAg-positive. HBV RT/HBsAg sequencing detected HBV genotype F2, with 2 immune-escape mutations in HBsAg (R122K and T140S) (Table 4).

The patient was lost to follow-up, so it was not possible to recover any additional information about treatment or the clinical course.

342

343 **Discussion**

344 This study documented a 3.5% incidence of HBV reactivation in an HSCT population
345 undergoing extensive LMV prophylaxis for the presence of any markers of HBV infection
346 or because the donors were anti-HBc+/-anti-HBs positive. This study also demonstrated
347 that a further 0.6% risk of HBV reactivation remained in an HSCT population who were not
348 considered at risk for HBV reactivation due to the absence of HBV markers. Using a
349 competing risk survival analysis, the cumulative probability of transplant-related mortality
350 or death due to haematological relapse was 45.5% at 6 years from transplantation, and the
351 cumulative probability of HBV reactivation at 6 years from transplantation was 15.8%.

352 All of the viral strains circulating in this population of HSCT patients harboured mutations
353 in the immune-active region of HBsAg, with only one exception, which reinforces the role
354 of HBsAg genetic variability in driving HBV reactivation that was highlighted in recent
355 studies (35-38).

356 Previously published data indicated an incidence of HBV-related hepatitis of up to 50%
357 (range: 3%-50%) in anti-HBc-positive patients undergoing HSCT (38-41). Giaccone L. and
358 collaborators (42), comparing two populations of allo-HSCT, anti-HBc-positive patients
359 who were treated or not treated with LMV, reported a significant risk of HBV reactivation (3
360 out of 14, 21.4%) in the untreated group.

361 The latest EASL guidelines (2012) recommend LMV prophylaxis in anti-HBc-positive
362 HSCT recipients, and the recent published ECIL-5 guidelines encourage the use of HBV
363 prophylaxis in HBV-negative patients receiving HSCs from anti-HBc-positive donors with a
364 level of evidence of A III (33, 43). The presence of anti-HBc in liver donors is a well-
365 recognized marker of the risk of HBV transmission and reactivation in liver transplantation

366 recipients (44). Conversely, little is known about the effects of anti-HBc-positive donors on
367 HBV-negative HSCT recipients. Recent reports documented the absence of HBV
368 transmission in HBV-naïve children undergoing prophylaxis with vaccination and/or
369 immunoglobulins when receiving HSCs from an anti-HBc-positive donor (45). Another
370 study demonstrated a protective role of HBV-immune/exposed HBV donors for anti-HBc-
371 positive HSCT recipients who did not receive LMV prophylaxis (21). However, HBV-
372 negative recipients receiving HSC from anti-HBc-positive donors had a 10.5% risk of
373 developing HBV-related hepatitis after HSCT compared with recipients from HBV-negative
374 donors in a country with a high level of HBV prevalence (46). Stem cells from HBV-positive
375 individuals were also shown to be a potential source of HBV (30). In our study, 11 out of
376 15 allo-HSCT HBV-negative patients with an anti-HBc-positive/HBsAg-negative donor
377 were treated with LMV. One of the remaining 4 patients, who was not treated with LMV,
378 experienced HBV reactivation. This result does not allow us to draw definitive conclusions;
379 however, the possible transmission of HBV infection from an OBI donor could not be
380 excluded. An OBI prevalence of 15.4% was documented in HSCT donors in Hong Kong
381 (46), but there is a lack of data on this issue in countries with low endemicity. Thus, the
382 issue of LMV prophylaxis in HBV-negative HSCT recipients from anti-HBc-positive donors
383 deserves further attention, and the use of more sensitive quantitative HBV-DNA assays
384 would be advisable in an HSCT setting to reduce the risk of HBV transmission (46).

385 Two patients (n. 3, an auto-HSCT patient, and n. 4, an allo-HSCT recipient from an HBV-
386 negative donor) were negative for any HBV markers and reactivated HBV infection. The
387 probability of HBV transmission by transfusion in these 2 cases seems negligible or very
388 low, due to the reduced residual risk (approximately 1:500,000 to 1:1,000,000) that is
389 actually linked to blood transfusion and to the progressive reduction of HBV infection
390 among Italian first-time blood donors (47). These 2 cases were more likely to be OBI

391 subjects, in 20% of which the anti-HBV antibodies progressively disappeared over time
392 (48). The OBI condition is very frequent in haematological patients, with a higher
393 prevalence in chronic lymphoid leukaemia subjects, which is approximately three-fold
394 higher than the prevalence observed in age- and sex-matched control subjects (28).
395 Nested PCR, real-time PCR or nucleic acid testing could more accurately quantify the
396 limited amount of HBV-DNA in patients with OBI. Therefore, these assays may be more
397 suitable in this category of patients to reduce the risk of HBV reactivation (46, 48).

398 In our study, the majority of HBV isolates obtained during the reactivation episodes
399 exhibited genotypic mutations that are associated with HBV immune escape. It has been
400 postulated that immune escape mutations may favour HBV reactivation during the
401 progressive weakening of the immune system (35, 37, 38, 49). The gene encoding HBsAg
402 overlaps with the gene encoding reverse transcriptase due to the peculiar organization of
403 the HBV genome. Therefore, some immune escape mutations may correspond to
404 mutations in the HBV reverse transcriptase enzyme that may potentially restore viral
405 replication impaired by LMV-resistance mutations (50).

406 In our study, 1 out of 4 patients (patient 2) was HBsAg-negative at the time of HBV
407 reactivation. This is in line with recently published studies showing that a substantial
408 proportion (ranging from 10% up to 80%) of patients remained HBsAg-negative despite the
409 reinitiation of viral replication (35, 36). The T123N HBsAg mutation, which was detected in
410 this patient, is known to introduce a new N-linked glycosylation site that can hamper
411 HBsAg recognition and quantification by the currently available diagnostic tests (35).

412 An LMV-resistant isolate was detected in one of the 47 at-risk patients who were treated
413 with LMV (Patient 1) for 41 months. The rate of developing LMV resistance increases with
414 the duration of its use, and the duration of LMV delivery is not fully defined in HSCT, where

immune recovery could take more than 2 years. In contrast, the cumulative probability of HBV reactivation has been reported to increase from 9% at 1 year up to 43% at 4 years after transplantation (51), and our study observed a similar increase (Fig. 1).

Before we can draw conclusions, some limitations of our study need to be discussed. The retrospective and nonrandomized nature of the study is its major limitation. In fact, given to the lack of standardized HBV infection monitoring, it is likely that episodes of recurrent HBV viremia in the absence of overt clinical hepatitis have been lost. However, before the prophylaxis was suspended, all patients were regularly tested for the presence of HBV serological markers and HBV-DNA, and no case of HBV reactivation was observed at the time of LMV suspension. The limited number of cases of HBV reactivation is an additional limitation because it does not permit us to draw definite conclusions. Furthermore, all patients were from a single institution, which might reduce the generalizability of the conclusions.

In conclusion, LMV prophylaxis has been demonstrated to have a protective role in preventing HBV reactivation in HSCT patients with active or resolved HBV infection and in HBV-negative recipients receiving HSCs from an anti-HBc-positive donor. The risk of reactivation persists in OBI patients and in those who undergo prolonged LMV prophylaxis. Notably, not all patients who were considered at risk of HBV reactivation received prophylaxis in our carefully evaluated HSCT population. This finding supports the hypothesis that the evaluation of immunocompromised patients with respect to HBV infection is a challenge, and a close collaboration among specialists would ensure better monitoring.

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Author contributions

Cerva C., Colagrossi L., Svicher V. and Sarmati L. contributed to the design of the study and drafting the article.

Maffongelli G., Malagnino V., Bianchi A., Ricciardi A., Picardi A., Cudillo L., Cerretti R., De Angelis G., and Cantonetti M. contributed to patient enrolment and acquisition of the data.

Salpini R., Battisti A., Pollicita M., Cerva C., Colagrossi L., Svicher V. and Sarmati L. contributed to virological studies and result analysis.

Di Carlo D. contributed to statistical data evaluation.

Andreoni M., Perno C.F., Arcese W., Svicher V. and Sarmati L. contributed to critically revising the article and the final approval of the version to be submitted.

458

459 **Figure legends**

460 **Figure 1. Survival analysis by competing risk estimates of the cumulative**
461 **probability of HBV reactivation.** The cumulative probability of HBV reactivation was
462 evaluated in the group of 59 patients 6 years from HSCT. Events of HBV reactivation,
463 transplant-related mortality and death for haematological relapse were evaluated.

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664

665

666 **Table 1. Patients' Characteristics**

Characteristics	Number of patients (%)
Total	**373**
Male	195 (52.3)
Median age (IQR), years	47 (35-55)
Diagnosis	
Acute Myeloid Leukaemia	127 (34.0)
Non-Hodgkin Lymphoma	79 (21.2)
Multiple myeloma	58 (15.6)
Acute Lymphoid Leukaemia	47 (12.6)
Hodgkin Lymphoma	36 (9.6)
Myelodysplastic syndrome	9 (2.4)
Chronic Myeloid Leukaemia	7 (1.9)
Myelofibrosis	6 (1.6)
Chronic Lymphoid Leukaemia	3 (0.8)
Aplastic anaemia	1 (0.3)
Transplant	
Autologous	117 (31.4)
Allogeneic	256 (68.6)
HLA haploidentical related donors	75 (29.3)
HLA identical sibling	99 (38.7)
HLA matched unrelated donor	79 (30.9)
HLA syngeneic	3 (1.2)
Acute GvHD[a] (N=256)	
Grade 0	167 (65.3)
Grade 1	39 (15.2)
Grade 2	35 (13.7)
Grade 3	6 (2.3)
Grade 4	9 (3.5)
Chronic GvHD[a] (N=256)	
Limited	35 (13.7)
Extensive	31 (12.1)
Allogeneic Conditioning (N=256)	
Myeloablative (MAC)	179 (70.0)
Reduced intensity (RIC)	77 (30.0)

667

668 [a] The percentage was calculated from 256 patients undergoing allogeneic HSCT

669

670 **Table 2: HBV serological profiles in HSCT recipient and donor subjects**
671

Recipient's serological HBV status	Autologous transplantation N (%)	Allogeneic transplantation N (%)	Donor's serological HBV status, N (%)[a]		
			Anti-HBc ± anti-HBs pos	Isolated anti-HBs pos	Neg for all HBV markers
Overall	117	256	25 (9.8)	50 (19.5)	181 (70.7)
Anti-HBc ± anti-HBs positive	10 (8.5)	27 (10.5)	10 (37.1)	5 (18.5)	12 (44.4)
Inactive carrier [b]	2 (1.7)	3 (1.2)	0	0	3 (100)
Isolated anti-HBs positive	23 (19.7)	110 (43.0)	8 (7.2)	30 (27.3)	72 (65.5)
Negative for all HBV markers	82 (70.1)	116 (45.3)	7 (6.1)	15 (12.9)	94 (81.0)

672

673 [a] The percentage was calculated in relation to each category of the recipient's serological HBV status

674 [b] Inactive carriers were defined as HBsAg-positive patients with serum HBV-DNA levels <2.000 IU/mL,
675 normal transaminase levels and the absence of clinical symptoms.

676

677

678 **Table 3: Incidence of HBV reactivation according to the presence or absence of HBV markers at pre-**
679 **transplant screening**
680

	N_{tot}	Use of LMV N (%)	Incidence of HBV reactivation N (% [95% C.I.])[a]
Anti-HBc-positive in the recipient and/or donor[a]	57	47 (82.5)	2 (3.5 [0.4%-12.1%])
Isolated anti-HBc-positive recipient	6 (10.5)	3 (50)	0
Anti-HBc + anti-HBs positive recipient	31 (54.4)	28 (1.1)	1
Inactive carrier recipient	5 (8.8)	5 (100)	0
HBV-negative allo-HSCT with an anti-HBc-positive donor	15 (26.3)	11 (23.4)	1
No positivity for anti-HBc in the recipient and/or donor	320	0	2 (0.6 [0.1%-2.2%])

681

682 [a] The percentages and the 95% C.I. were calculated by stratifying the patients according to their HBV
683 serological profiles

684

685

686 **Table 4: Clinical and virological parameters of the 4 HBV-reactivated patients**

ID_Patient	1	2	3	4
Clinical characteristics				
HBV serological status [a]	Anti-HBc and anti-HBs positive	Negative	Negative [a]	Negative
N of HSCT before HBV-R	2	1	2	1
HSCT Type	Autologous/ Allogeneic	Allogeneic	Autologous/ Allogeneic	Autologous
Donor's HBV serological status [a, b]	N.A./anti-HBc and anti-HBs positive	anti-HBc alone	N.A./ Negative	N.A.
Months from disease diagnosis to HBV-R	71	36	39	62
Months from first HSCT to HBV-R	41	30	12	52
Outcome	-	Dead	Dead	-
GVHD concomitant	Yes	No	Yes	
LMV prophylaxis	Yes (41 months)	No	No	No
Virological characteristics				
Genotype	D	D	D	F
HBV-DNA Log (IU/ml)	5.97	3.24	5.08	8.94
ALT (U/L)	31	2,159	361	61
AST (U/L)	29	2,570	203	31
HBsAg quantitative (IU/ml)	>52,000	Negative	4,029	>52,000
HBsAg mutations associated with immune escape	None	V96A/V, M103I, T123N, C124Y, T126I, G145 K/R	D144E	R122K, T140S
RT mutations associated with drug resistance	L80I	A181S, V214A	None	None

687

688 [a] Negative indicates negativity for all HBV serological markers

689 [b] The donor status is defined as not applicable when the transplanted patient received an autologous transplantation

690 N.A., Not applicable

691

692

693