Received: 2 April 2020 | Revised: 27 November 2020 | Accepted: 8 December 2020

DOI: 10.1111/ajt.16450

BRIEF COMMUNICATION

AJT

Positive HCMV DNAemia in stem cell recipients undergoing letermovir prophylaxis is expression of abortive infection

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Funding information
Ministero della Salute, Fondazione IRCCS Policlinico San Matteo, Ricerca Corrente, Grant/Award Number: 80720 and 80207

Abstract

Letermovir (LMV) inhibits HCMV replication by binding to components of the HCMV-terminase complex showing a potential role in prevention of HCMV-related complications in allogenic hematopoietic stem cell transplant recipients (allo-HSCTRs). However, little is known about breakthrough HCMV infection and the relevance of HCMV DNAemia during prophylaxis. We reported the results of a multicenter prospective study involving five Italian centers in the management of HCMV DNAemia in 75 adult HCMV-seropositive allo-HSCTRs undergoing LMV prophylaxis. The aim of the present study was to characterize the presence of real HCMV reactivation during LMV prophylaxis. Then, the presence of circulating infectious HCMV particles was determined by virus isolation and degradation of free-floating viral DNA. This report provides the first evidence that during LMV prophylaxis the clinical relevance of HCMV DNAemia should be critically considered.

KEYWORDS
clinical research/practice, infection and infectious agents – viral: Cytomegalovirus (CMV), infectious disease, molecular biology, molecular biology: DNA, translational research/science

Abbreviations: ALC, absolute leukocytes count; allo-HSCTRs, allogenic hematopoietic stem cell transplant recipients; ANC, absolute neutrophils count; CDV, cidofovir; FOS, foscarnet; GCV, ganciclovir; HCMV, human cytomegalovirus; LMV, letermovir; PBLs, peripheral blood leukocytes; PCR, polymerase chain reaction; PET, preemptive treatment; VGCV, valganciclovir.

1 | INTRODUCTION

Human Cytomegalovirus (HCMV) remains one of the major viral opportunistic infections in allogenic hematopoietic stem cell transplant recipients (allo-HSCTRs), despite the availability of efficient diagnostic and therapeutic approaches.[1] Although available anti-HCMV drugs may be effective for HCMV prophylaxis in HSCT, the use of ganciclovir (GCV) and valganciclovir (VGCV) is currently limited for clinically significant myelosuppression while foscarnet (FOS) and cidofovir (CDV) are commonly associated with nephrotoxicity.[2] Interestingly, a double-blind, placebo-controlled, randomized trial performed in 184 allo-HSCTRs revealed the non-superiority of VGCV prophylaxis in comparison to PCR-guided preemptive therapy (PET).[3] Thus, in the last 20 years, PET strategy has been used as standard of care in allo-HSCTRs.[4] In Italy, although differences among transplant centers in the cut-off levels for starting preemptive treatment in allo-HSCTRs still remain, a consensus statement has been published indicating that GCV or VGCV-PET should be administered when in presence of HCMV DNAemia higher than 1000 copies/ml plasma or 10 000 copies/ml whole blood.[5]

The recent approval of Letermovir (LMV) for prophylaxis in allo-HSCTRs has changed the scenario, opening new issues in the field of HCMV prevention.[6] A phase III clinical trial in allo-HSCTRs revealed the efficacy of prophylactic treatment with LMV (62/325 19.1% HCMV DNAemia positive in LMV arm vs 85/170; 50% in placebo arm, $p < .001$), with an excellent safety profile. Nevertheless, in the presence of positive HCMV DNAemia (higher than 150 copies/ml in plasma samples) GCV-PET was initiated in 24 patients (7.7%).[6]

LMV inhibits the terminal phase of HCMV replication by targeting the HCMV-terminase complex (pUL56, pUL89, and pUL51) instead of viral DNA polymerase (pUL54).[7,8] Therefore, a very late stage of HCMV replication is inhibited and long DNA concatemers are not cleaved into single viral subunit, producing non–infectious long DNA molecules.[9] In other words, with respect to untreated cells, the same amount of viral DNA is produced but no infectious particles are packed and released from (now) abortively infected cells.

To date, no data are available about the clinical relevance of HCMV DNAemia in patients undergoing prophylaxis with LMV. Indeed, non–infectious HCMV DNA released from degrading abortively infected cells could be detectable in blood by current high sensitivity real-time polymerase chain reaction (PCR) methods in blood samples, even in the absence of infectious virions. Thus, a potential misinterpretation of PCR results during LMV prophylaxis is predictable.

The objective of this multicenter prospective study was to describe a real-life experience of five Italian centers in the management of the first cohort of allo-HSCTRs with undetectable HCMV DNA in whole blood during the 5 days prior to enrolment and undergoing prophylaxis with LMV. The aim of the study was to dissect between non–infectious and infectious HCMV DNAemia using additional diagnostic methods.

2 | PATIENTS AND METHODS

Prophylaxis with Letermovir was administered in 77 consecutively enrolled HCMV-seropositive patients with undetectable HCMV DNAemia in the prior 5 days. All the patients were enrolled in five Italian centers (23 from IRCCS Policlinico San Matteo, Pavia; 22 from IRCCS San Raffaele, Milano; eight from Spedali Civili, Brescia; 14 from Policlinico Sant'Orsola, Bologna and 10 from Policlinico Umberto I). Two patients out of 77 were treated for positive HCMV DNA before the assessment of HCMV viremia and HCMV DNA in plasma and were excluded from the subsequent analyses. Characteristics of the 75 analyzed patients, including underlying diseases, therapies and demographic data are given in Table 1.

All the patients gave their written informed consent and the study was performed according to the guidelines of the Institutional Review Board of the Fondazione IRCCS Policlinico San Matteo (protocol no. 20200013139).

Prophylaxis with a standard dose of 480 mg of LMV daily or 240 mg of LMV daily if co-administered with cyclosporine A was used. According to approved therapeutic protocol, prophylaxis was started by 28th day after transplant and lasted for 100 days. All the patients were monitored twice a week for the first month and then weekly for the following period of prophylaxis for the quantification of HCMV DNAemia in whole blood and, in case of positive results, in plasma using standardized methods.[10,11]

All the patients with positive HCMV DNAemia were referred at IRCCS Policlinico San Matteo and Policlinico Sant'Orsola for further characterization as described below. In keeping with the Italian consensus paper LMV should have been shifted to GCV in presence of HCMV DNAemia higher than 10 000 copies/ml whole blood. As an additional safety rule, the shift to GCV was contemplated when in the presence of ascertained HCMV replication.

2.1 | Characterization of true vs abortive HCMV reactivation during LMV prophylaxis: (i) virus isolation from peripheral blood leucocytes (PBLs)

HCMV was isolated from circulating PBLs in shell vial cultures using a quantitative assay previously described.[12,13] The load of infectious virus in whole blood samples was determined by counting the number of fluorescent fibroblast nuclei (viremia).[12]

2.2 | Characterization of true vs abortive HCMV reactivation during LMV prophylaxis: (ii) quantification of encapsidated vs free-floating HCMV DNA in blood

The amount of free-floating viral DNA from degrading cells with respect to the amount of encapsidated DNA was determined in plasma samples. In detail, the aliquot protected by the viral capsid

TABLE 1 Demographic and clinical characteristics of enrolled patients

Characteristics	Number (%)
Age (years; median [IQR])	58 [48–64]
Male sex	49 (65.3%)
Donor HCMV serostatus (D−/R+)	28 (37.3%)
Underlying diagnosis	
Leukemia	47/75 (62.7%)
Lymphoma	6/75 (8.0%)
MDS/MPN	16/75 (21.3%)
Myelofibrosis	4/75 (5.3%)
Myeloma	2/75 (2.7%)
Conditioning regimen	
Busulfan-Fludarabine	5/75 (6.7%)
Busulfan-PT-Cy	3/75 (4%)
Busulfan-thiotepa-fludarabine	35/75 (46.5%)
Melphalan-thiotepa-fludarabine	5/75 (6.7%)
Treosulfan-fludarabine	5/75 (6.7%)
Treosulfan-melphalan-fludarabine	11/75 (14.7%)
Others	11/75 (14.7%)
Type of donor	
HLA-matched related	11/75 (14.7%)
Haploidentical	20/75 (26.7%)
Matched/mismatech unrelated donor	41/75 (54.6%)
Umbelical cord blood	3/75 (4%)
GvHD prophylaxis	
Cyclosporine A-MTX	18/75 (24%)
Cyclosporine A-MTX-MMF/ATG	16/75 (21.3%)
Cyclosporine A-PT-Cy-MMF	12/75 (16%)
PT-Cy-sirolimus-MMF	13/75 (17.4%)
Others	16/75 (21.3%)
100 days-all cause mortality	5/75 (6.7%)
ANC > 500 (days; median [IQR])	18 [15–23]
HCMV positive DNAemia (whole blood)	26/75 (34.7%)
Start LMV prophylaxis (median (IQR); days post-tx)	4 (1–13.5)
Median period of follow-up (median (IQR); days post-tx)	105 (101–113.8)

Abbreviations: ANC, absolute neutrophils count; ATG, anti-thymocyte globulin; D, donor, HCMV, human cytomegalovirus; IQR, interquartile range; LMV, letermovir; MDS/MPN, myelodysplastic/myeloproliferative neoplasms; MMF, mycophenolate mofetil; MTX, methotrexate; Pt-Cy, Cyclophosphamide; R, recipient; tx, transplant.

and resistant to degradation by DNAse I was subtracted from the total plasma DNA value.[14] To elaborate, 100 µl of undigested sample was processed in parallel, omitting the DNAse and the reaction buffer from the mixture. DNA was then extracted with NUCLISENS MINIMAG (bioMerieux) and was eluted in 25 µl of distilled water. As control, naked linear plasmid at standard concentration was used. Finally, HCMV real-time PCR was performed as previously reported.[15]

3 | RESULTS AND DISCUSSION

Given the higher sensitivity of whole blood DNAemia, 26/75 (34.7%) patients showed at least one positive HCMV DNAemia measurement in whole blood samples, while 21/75 (28%) showed at least one positive HCMV DNAemia measurement in plasma samples. Of the 26 whole blood DNAemia-positive samples, seven (26.9%) showed a single detectable positive result during LMV prophylaxis, while the other 19 (73.1%) had multiple DNAemia-positive samples. Kinetics of HCMV DNAemia in whole blood and in plasma of the patients with multiple HCMV DNAemia is described in Figure 1.

Elaborately, the first positive HCMV DNAemia in whole blood was observed at median 7 days after starting prophylaxis [IQR 2.0–13 days], the median peak of HCMV DNAemia was 1179 copies/ml [IQR 300–1.710 copies/ml] (471 UI/ml IQR 120–684 IU/ml) reached at median day 4 [IQR 1.0–8.0 days]. Interestingly, we observed that among the 27/75 (36%) patients who started prophylaxis earlier (day 0–1) only four developed at least one positive HCMV DNAemia event (14.8%); on the other hand among the 48/75 (64%) patients who started LMV after the first day posttransplant (median 10 days, range 2–27 days), 22 (45.8%) developed at least one positive HCMV DNAemia during the prophylaxis period (p = .0107). On the other hand, no difference in terms of positive HCMV DNAemia events was observed between the group of HCMV D−/R+ and HCMV D+/R+ subjects since 10/28 (35.7%) HCMV D−/R+ allo-HSCTRs and 16/47 (34.0%) HCMV D+/R+ allo-HSCTRs reported at least one positive HCMV DNAemia event (p = .9999).

Finally, in terms of LMV dose, in 51/75 (68%) patients a daily dose of 240 mg (240-dosegroup) was administered while a daily dose of 480 mg was used in the remaining 24/75 (32%) patients (480-dosegroup), according to the previously specified criteria. No difference was observed in the number of positive HCMV DNAemia events according to LMV dose administered. To be elaborate, at least one positive HCMV DNAemia event was observed in 19/32 (37.3%) patients out of the 240-dose group and in 7/24 (29.2%) patients out of the 480-dose group (p = .6061).

HCMV plasma DNAemia after digestion with DNAse I was undetectable in all patients, suggesting the absence of replicative HCMV DNA. Similarly, in none of the patients HCMV could be isolated in shell vial cultures, further corroborating the finding of abortive HCMV replication during LMV prophylaxis. For this reason, no patients received GCV-PET.

Interestingly, according to current standards of care, all patients with positive HCMV DNAemia should have been switched to GCV-PET. In addition, if adopting the suggested cut-off of 1000 HCMV DNA copies/ml plasma, about half of the patients (6/15, 40%) would have been switched, while if using the cut-off value of 10 000 copies/ml whole blood none would have been submitted to unjustified GCV treatment (Figure 1).

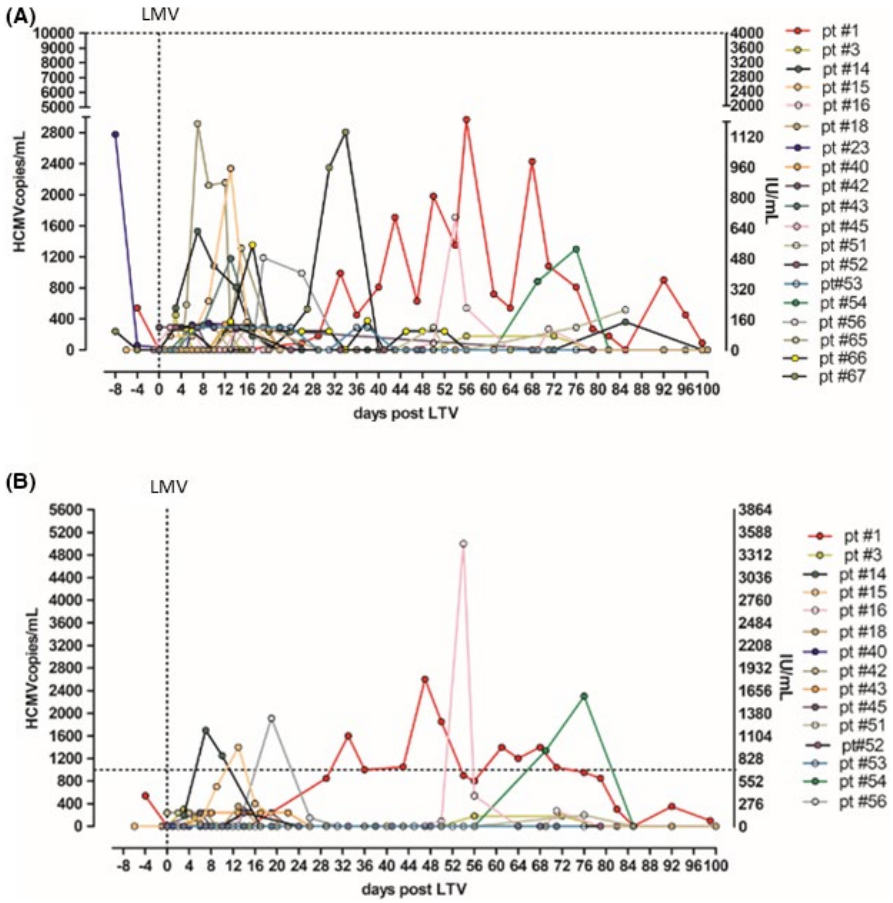

FIGURE 1 Kinetics of HCMV DNAemia in whole blood (A) and plasma samples (B) for 19 and 15 patients, respectively, are described. The dotted horizontal lines indicate two traditional thresholds of HCMV DNA used for starting preemptive therapies (10 000 copies/ml and 1000 copies/ml in whole blood (A) and in plasma (B), respectively). Both copies/ml and IU/ml are displayed in the graphs

In Figure 2, three exemplifying cases are shown. For each patient, white blood cell and platelet reconstitution as well as HCMV DNAemia measured in whole blood and plasma samples are described.

In this brief report, we described the first Italian real-life experience in allo-HSCTRs prophylaxis using the new anti-HCMV drug LMV. Although the drug has been approved for excellent safety profile as well as for efficacy in preventing clinically significant HCMV infection,[6] the clinical impact of detectable HCMV DNA during the period of prophylaxis has not been clarified yet. We observed that about one-third of the patients developed at least one positive HCMV DNAemia on whole blood samples, and about 28% had positive plasma HCMV DNAemia despite prophylaxis.

According to registrative suggestions, patients with positive HCMV DNAemia should be switched to GCV-PET. However, the mechanism of action of LMV led us to suspect that some patients could be switched to GCV-PET because of detection of non–replicative HCMV DNA in blood, thus exposing them to unjustified detrimental effects of GCV on engraftment and immunologic reconstitution. According to this hypothesis, monitoring of HCMV DNAemia was implemented in both whole blood and plasma, in addition with the measurement of HCMV DNAemia on plasma samples after DNAse digestion and shell vial HCMV isolation. All patients with positive HCMV DNAemia measured either in whole blood and plasma had negative plasma DNAemia following DNAse I digestion as well as negative shell vial isolation. These results suggested that circulating HCMV DNA during LMV prophylaxis was a noninfectious material released by abortively infected cells rather than expression of productive infection. The possible explanation is related to the mechanisms of action of LMV, which does not inhibit viral DNA synthesis but its further maturation into individual genomes.[16,17]

In this new scenario of anti-HCMV prophylaxis, conventional HCMV monitoring approaches might be revised and reinterpreted according to the LMV mechanism of action. It is conceivable that HCMV DNA positive results during LMV prophylaxis should be further analyzed and characterized before switching to GCV-PET, especially in the early phases of post–transplant hematologic recovery.

Of note is the finding that none of the patients during LMV prophylaxis reached the cut-off of 10 000 HCMV DNA copies/ml whole blood,[5] while relatively high plasma values were more commonly observed. Based on HCMV DNAemia observed in plasma samples, about half of the analyzed patients should have received an unjustified GCV-PET treatment. A possible explanation is the observed accumulation of HCMV DNA in plasma in the descending phases of infection as a result of cell degradation.[18] Finally, abortive HCMV DNAemia was more frequently observed in patients with delayed prophylaxis initiation. This finding could be possibly associated with a higher chance of initial viral reactivation and consequently higher chance to detect viral DNA concatemers.

The major strength of this report is that we provided a detailed method for the characterization of abortive HCMV DNAamia during LMV prophylaxis, using both molecular and cellular approaches in order

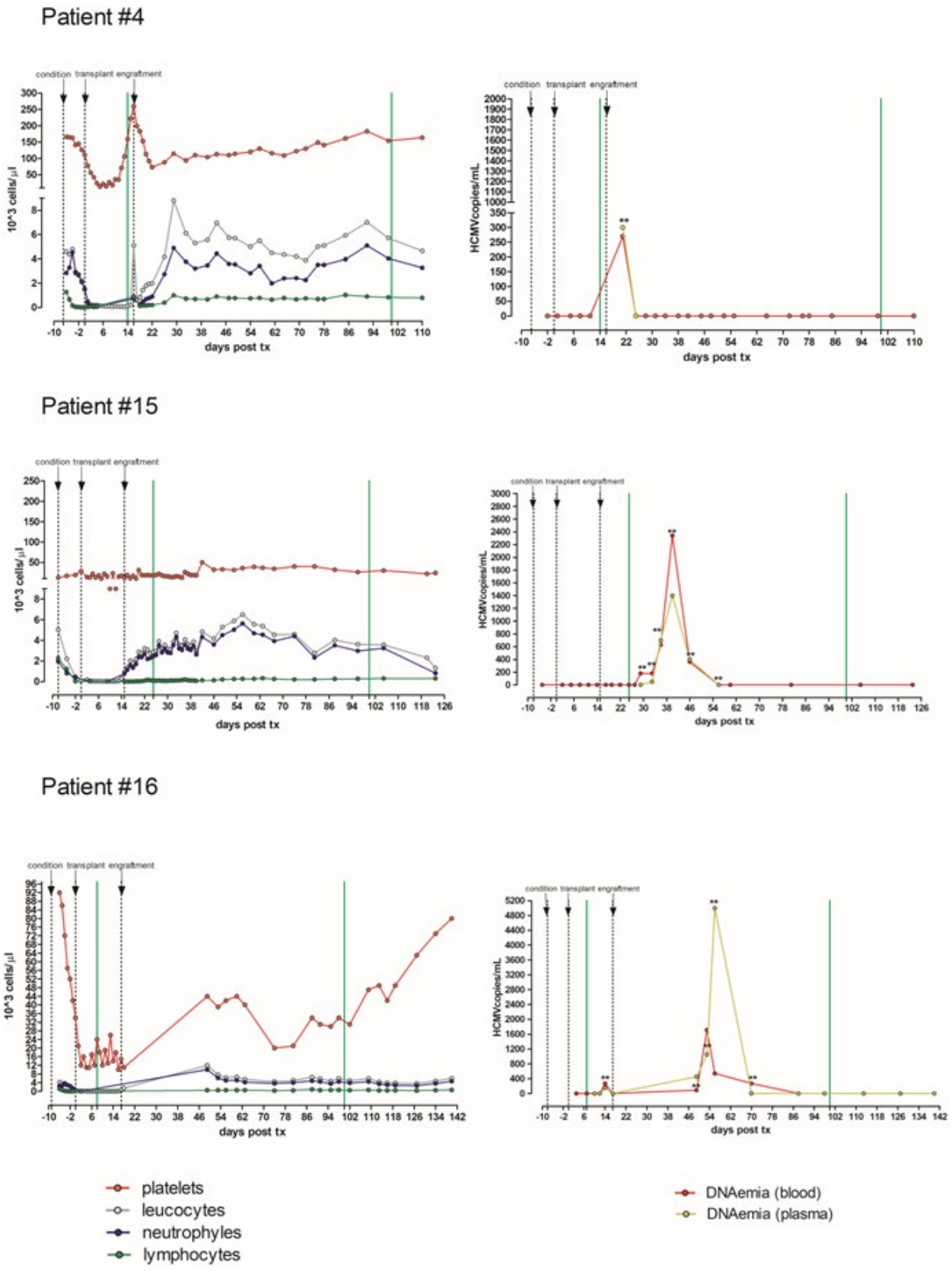

FIGURE 2 Three exemplifying cases are shown. For each patient, white blood cell and platelet reconstitution (on the left) and HCMV DNAemia in whole blood and plasma samples (on the right) are shown. The vertical green lines indicate the period of letermovir prophylaxis. Tx, transplant. **HCMV viremia and HCMV DNAemia after DNAse digestion negative

to exclude the presence of viral DNA present in whole viral particles. Since cultures from PBL are less sensitive than PCR potential false-negative results were taken into account. For that reason, molecular and cellular assays were always performed in parallel. Although the study was performed in a small sample setting, we observed a high reproducibility of the methods between the different participating units.

In summary, we demonstrated that positive HCMV DNAemia both in whole blood and plasma detected during LMV prophylaxis in allo-HSCTRs might not be associated to a complete replication cycle. Thus, to dissect between real reactivations and abortive infections additional assays, including molecular and cellular approaches, could be useful. On the other hand, a practical and safe approach for starting GCV-PET in patients undergoing LMV prophylaxis could be to consider a cut-off of 10 000 HCMV DNA copies/ml whole blood, as suggested by the last consensus Italian conference[5] and never reached in patients with abortive HCMV DNAemia during LMV prophylaxis.

Finally, an earlier prophylaxis initiation is advised, in order to avoid these false-positive DNA results. Naturally, a prospective trial proving the advantage of this approach is needed.

ACKNOWLEDGMENTS

We thank Daniela Sartori for manuscript editing. This study was supported by grants from the Ministero della Salute, Fondazione IRCCS Policlinico San Matteo, Ricerca Corrente grant no. 80720 (to A.A.C) and grant no. 80207 (to F.B.).

DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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How to cite this article: Cassaniti I, Colombo AA, Bernasconi P, et al. Positive HCMV DNAemia in stem cell recipients undergoing letermovir prophylaxis is expression of abortive infection. *Am J Transplant*. 2021;00:1–7. https://doi.org/10.1111/ajt.16450