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RESEARCH ARTICLE

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Monitoring of plasma Torque teno virus, total *Anelloviridae* and Human Pegivirus 1 viral load for the prediction of infectious events and acute graft versus host disease in the allogeneic hematopoietic stem cell transplantation setting

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Abstract

Anelloviridae and Human Pegivirus 1 (HPgV-1) blood burden have been postulated to behave as surrogate markers for immunosuppression in transplant recipients. Here, we assessed the potential utility plasma Torque teno virus (TTV), total *Anelloviridae* (TAV), and HPgV-1 load monitoring for the identification of allogeneic hematopoietic stem cell transplantation recipients (allo-HSCT) at increased risk of infectious events or acute graft versus host disease (aGvHD). In this single-center, observational study, plasma TTV DNA, TAV DNA, and HPgV-1 RNA loads were monitored in 75 nonconsecutive allo-HSCT recipients (median age, 54 years). Monitoring was conducted before at baseline or by days +30, +60, +90, +120, and +180 after transplantation. Pneumonia due to different viruses or *Pneumocystis jirovecii*, BK polyomavirus-associated haemorrhagic cystitis (BKPyV-HC), and Cytomegalovirus DNAemia were the infectious events considered in the current study. Kinetics of plasma TTV, TAV DNA, and HPgV-1 RNA load was comparable, with though and peak levels measured by days +30 and day +90 (+120 for HPgV-1). Forty patients (53%) developed one or more infectious events during the first 180 days after allo-HSCT, whereas 29 patients (39%) had aGvHD (grade II–IV in 18). Neither, TTV, TAV, nor HPgV-1 loads were predictive of overall infection or CMV DNAemia. A TTV DNA load cut-off ≥4.40 $\log_{10}$ (pretransplant) and ≥4.58 $\log_{10}$ (baseline) copies/mL predicted the occurrence of BKPyV-HC (sensitivity ≥89%, negative predictive value, ≥96%). TTV DNA loads ≥3.38 $\log_{10}$ by day +30 anticipated the occurrence of aGvHD (sensitivity, 90%; negative predictive value, 97%). Pretransplant HPgV-1 loads were significantly lower ($p = 0.03$) in patients who had aGvHD than in those who did not. Monitoring of TTV DNA or HPgV-1 RNA plasma levels either before or early after transplantation may be ancillary to identify allo-HSCT recipients at increased risk of BKPyV-HC or aGvHD.

KEYWORDS
aGvHD, BK-virus cystitis, Cytomegalovirus DNAemia, Human Pegivirus, infection, total *Anelloviridae*, TTV, viral load

1 | INTRODUCTION

The *Anelloviridae* family includes circular, negative sense, single-stranded DNA viruses, of which three genera, *Alphatorquevirus* (Torque teno virus [TTV]), *Betatorquevirus* (Torque teno minivirus [TTMV]) and *Gammatorquevirus* (Torque teno midi virus [TTMDV]) infect humans chronically.[1,2] The *Anelloviridae* represent a major fraction (around 70%) of the human blood virome that remains relatively stable over years in the healthy host.[3] The *Anelloviridae* are highly T-lymphotrophic and T-cell dependent immune mechanisms are mainly responsible of virus replication control; in this context, the *Anelloviridae* burden in the blood compartment was postulated to behave as a surrogate marker of the net state of immunosuppression in transplant recipients.[4] A large body of experimental evidence supports this assumption in the solid organ transplantation (SOT) setting[5,6]; in fact, high TTV DNA levels associates with the occurrence of infectious events, whereas the opposite holds true for acute rejection. The kinetics of TTV DNA load and its association with immune-related clinical events in the allogeneic hematopoietic stem cell transplantation (allo-HSCT) setting is more complex due to conditioning-driven lymphodepletion and the timing of lymphocyte reconstitution.[6] In this context, the kinetics of TTV DNA load in plasma appear to mirror that of immunological reconstitution early after allo-HSCT, whereas it is more reflective of the degree of immunosuppression at late times (over day +100) after transplantation.[6] Only a few studies have investigated the potential value of TTV DNAemia monitoring for the prediction of immune-related clinical events such as infection (i.e., Cytomegalovirus-CMV-DNAemia) or acute graft versus host disease (aGvHD) in the allo-HSCT setting, these yielding somewhat conflicting observations.[7–13] Co-detection of TTV, TTMV, and TTMDV in pretransplant and postengraftment plasma specimens was documented by our group using next generation sequencing in a large fraction of allo-HSCT recipients (more than two-thirds).[14] In view of this finding we developed a quantitative real-time polymerase chain reaction (PCR) assays targeting TTMV and TTMDV in addition to TTV (total *Anelloviridae*-TAV-) which we hypothesized might add value to TTV-specific PCR assays in the inference of the net state of immune competence in this clinical setting, although this assumption was not assessed.[14]

Human Pegivirus 1 (HPgV-1) is a 9.4 kb positive-sense single-strand RNA genome enveloped virus that belongs to the genus *Pegivirus* of the family *Flaviviridae*.[15] HPgV-1 is a lymphotropic virus that may establish a subclinical persistent infection in healthy individuals.[16] A number of studies have shown that HPgV-1 persistent infection may modulate host immune responses; in this sense, notably, HPgV-1 infection may downregulate immune activation in the HIV-1 infection setting.[16] HPgV-1 RNAemia is frequently detected in allo-HSCT recipients (ranging from 14% to 61%).[17–23] In a previous study, HPgV-1 RNAemia was associated with an impaired NK cell, but not T cell, immune-reconstitution[24]; yet, to date no impact of HPgV-1 RNAemia on clinical outcomes has been reported in the allo-HSCT setting.[18–23]

Here, we further characterized the kinetics TTV, TAV, and HPgV-1 viral load in plasma following allo-HSCT and investigated whether quantification of TAV, TTV, and HPgV-1 viral load at different time points would allow anticipation of the occurrence of immune-related clinical complications such as certain infections, whose control is mainly dependent on T-cell-mediated immune mechanisms, and acute graft versus host disease (aGvHD).

2 | MATERIALS AND METHODS

2.1 | Patients and samples

In this observational retrospective study, a total of 75 nonconsecutive adult patients (median age, 54 years; range, 19–70) who underwent T-cell replete allo-HSCT at the University Clinical Hospital of Valencia between 2017 and 2021 were included. Relevant clinical and demographic characteristics of the patients are displayed in Table 1. The only criterium for patient inclusion was the availability of at least 3 stored plasma samples (−80°C) collected at different time points before (7–10 days) at baseline (Day 0) or after (+30, +60, +90, +120, and +180) transplantation. Medical records were retrospectively reviewed for extraction of demographic and clinical data of interest (occurrence of certain infectious events and aGvHD) developing within the first 180 days after transplantation. Exemption of informed consent was obtained from the University Clinical Hospital Ethics Committee (authorization number 2019/246).

2.2 | Quantification of Torque teno virus DNA, total Anelloviridae DNA and Human Pegivirus 1 in plasma

Nucleic acid extraction was performed from 200 µL of plasma using the QIAsymphony platform (Qiagen). TTV DNA was quantified using a Taqman real-time PCR, as previously described.[7] TAV DNA was quantified using a SYBR Green based real-time PCR using the universal primers NG779/NG781 that target a conserved segment of the UTR region, as previously described.[14,25] Human Pegivirus (HPgV-1) RNA was quantified using a one-step RT-PCR as previously described.[26]

TABLE 1 Demographic and clinical characteristics of allogeneic hematopoietic stem cell transplant recipients included in the study.

Variable	No. (%)
Sex	
Male	46 (61)
Female	29 (39)
Underlying disease	
Acute myeloid leukemia	28 (37.3)
Acute lymphocytic leukemia	1 (1.3)
Chronic myeloid leukemia	3 (4)
Chronic lymphocytic leukemia	3 (4)
Hodgkin's lymphoma	13 (17.3)
Multiple myeloma	1 (1.3)
Myelodysplastic syndrome	4 (5.3)
Myelofibrosis	4 (5.3)
Non-Hodgkin's lymphoma	15 (20)
Other hematological diseases	3 (4)
Stem cell source	
PB	75 (100)
Donor type	
Related	46 (61)
Unrelated	29 (39)
HLA matching	
Matched	41 (55)
Mismatched	9 (12)
Haploidentical	25 (33)
CMV serostatus	
D+/R+	44 (59)
D−/R+	20 (27)
D+/R−	5 (6)
D−/R−	6 (8)
Conditioning regimen	
Myeloablative	14 (19)
Reduced intensity	61 (81)
aGvHD prophylaxis	
Sirolimus plus MMF plus cyclophosphamide	71 (94.6)
Tacrolimus plus sirolimus	2 (2.6)
Tacrolimus plus cyclophosphamide	2 (2.6)
Occurrence of aGvHD	
Grade I	11 (14.6)
Grades II–IV	18 (24)

Abbreviations: aGvHD, acute graft vs. host disease; CMV, cytomegalovirus; D, donor; HLA, human leukocyte antigen; MMF, mycophenolate mofetil; PB, peripheral blood R, recipient.

2.3 | Definitions and diagnostic criteria

Pneumonia caused by SARS-CoV-2 was defined as the presence of lower respiratory tract disease symptoms and pulmonary infiltrates by chest-X-ray or chest CT scan, along with the detection of SARS-CoV-2 RNA in respiratory samples by the TaqPath COVID-19 RT-PCR (ThermoFisher Scientific). Pneumonia caused by *Pneumocystis jirovecci* was defined as the presence of clinical symptoms with suggestive radiological findings and the presence of a positive PCR result (RealCycler PJIR kit®; Progenie Molecular) in bronchoalveolar lavage samples in the absence of an alternate diagnosis. Proven Cytomegalovirus (CMV) Pneumonia was diagnosed according to laboratory and clinical criteria previously reported by Ljungman et al.[27] CMV DNA was quantified in plasma samples by using the Abbott RealTime CMV Assay (Abbott molecular). BK polyomavirus-associated haemorrhagic cystitis (BKPyV-HC) was diagnosed according to ECIL clinical guidelines.[28] BK virus DNA was quantified in plasma samples by using the Realquality RQ-BKV (AB Analitica) PCR.

2.4 | Statistical analysis

Frequencies of categorical variables were compared using the Fisher's exact test, whereas medians of continuous variables were compared by means of the Man–Whitney *U* test. Correlation between continuous variables was carried out by the Spearman correlation test. Two-sided exact *p* values are reported, considering $p < 0.05$ statistically significant. The selection of the best cut-off in terms of sensitivity and specificity for a given variable was carried out by the receiver-operating characteristic (ROC) analysis. All analyses were performed using GraphPad Prism versión 9.0.

3 | RESULTS

3.1 | kinetics of Torque teno virus, total Anelloviridae and Human Pegivirus-1 load in plasma following allogeneic hematopoietic stem cell transplantation

A total of 364 plasma samples from 75 patients were available for viral loads quantitation, of which 52 were obtained before transplantation (preconditioning), 45 the day of transplantation (baseline), 64 on day +30, 65 on day +60, 61 on day +90, 44 on day +120, and 33 on day +180, in all cases from unique patients. Qualitative PCR results for all viruses at different time points on an individual basis are shown in Supporting Information: Table 1. The full set of specimens ($n = 7$) was available from 15 patients. While some of these patients displayed persistently positive ($n = 6$ for TTV, and $n = 3$ for HPgV-1) or negative ($n = 4$ for HPgV-1), other tested negative before transplantation and positive in one or more specimens afterwards ($n = 4$ for TTV and $n = 7$ for HPgV-1), this suggesting the occurrence of virus infection following transplantation. Interestingly, a number of

TABLE 2 Detection rate and plasma viral loads of Torque teno virus DNA, total *Anelloviridae* DNA, and Human Pegivirus-1 RNA at different time points prior or after allogeneic hematopoietic stem cell transplantation.

Time of viral load monitoring (no. of patients tested)	TTV detection (%), median viral load [IQR range] in log_{10} copies/mL	TAV detection (%), median viral load [IQR range] in log_{10} copies/mL)	HPgV-1 detection (%), median viral load [IQR range] in log_{10} copies/mL
Pretransplant (52)	77%, 4.15 [2.16–5.36]	43% 0 [0–3.20]	48% 0 [0–4.78]
Baseline (45)	78% 4.41 [2.96–5.68]	36% 0 [0–2.54]	42% 0 [0–4.01]
+30 (64)	63% 3.40 [0–4.58]	21% 0 [0–0]	51% 2.16 [0–4.31]
+60 (65)	94% 6.24 [4.61–7.50]	65% 3.0 [0–3.83]	56% 2.36 [0–4.52]
+90 (61)	100% 7.29 [6.28–8.11]	82% 3.97 [3.14–4.98]	59% 2.72 [0–4.67]
+120 (44)	96% 7.12 [5.50–8.22]	80% 3.61 [2.61–4.59]	64% 4.04 [0–5.35]
+180 (33)	97% 6.53 [4.85–8.25]	65% 2.63 [0–4.36]	56% 3.79 [0–5.02]

Abbreviations: HPgV-1, Human Pegivirus-1; TAV, total anelloviridae; TTV, Torque teno virus.

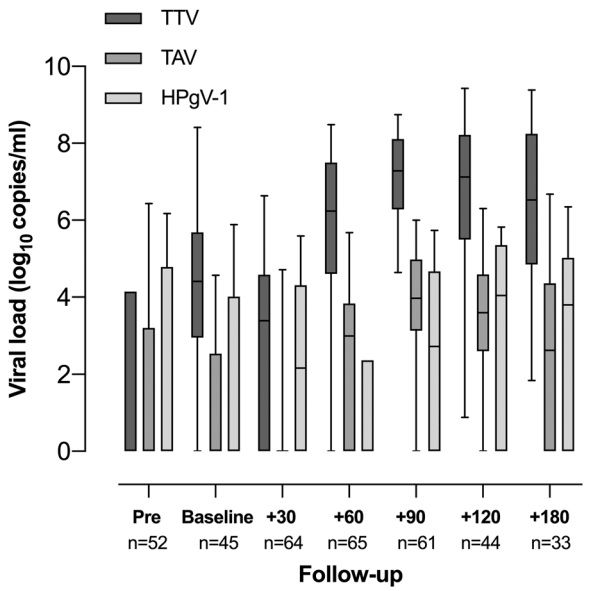

FIGURE 1 Plasma Torque teno virus, total *Anelloviridae* and Human Pegivirus 1 viral loads in allogeneic hematopoietic stem cell transplantation recipients over the study period. The number of available specimens at each monitoring time point is shown.

patients testing positive before conditioning turned out negative at baseline (4/30 for TTV, 5/13 for TAV and 1/14 HPgV-1) (Supporting Information: Table 1).

Most patients had TTV or TAV DNA detectable (in at least in one sample) over the study period (73/75; 97% and 64/75; 85%, respectively). As shown in Table 2, the rate of TTV and TAV detection throughout the study period followed a similar pattern; namely, it decreased by day +30 respect to that found at baseline (15% reduction), and subsequently increased reaching the maximum by day +90 (100% for TTV and 80% for TAV). Kinetic of plasma TTV and TAV DNA load, depicted in Figure 1, was comparable, with though and peak levels measured by days +30 and day +90, respectively (Figure 1 and Table 2). Although TTV and TAV viral loads correlated fairly well (Rho, 0.71; 95% CI, 0.66–0.76; $p < 0.001$-Supporting Information: Figure 1), the former were significantly higher than the latter (overall, median 5.96 log_{10} copies/mL; range, 1.11–9.64 vs. overall median 3.90 log_{10} copies/mL; range, 2.05–7.07; $p = 0.01$) at all time points (Table 2).

As shown in Table 2, HPgV-1 was detected in at leat one specimen from 50/75 patients (66%). The rate of HPgV-1 detection remained relatively estable throughout the study period (minimum 42% at baseline and maximum, 64% by day +120); nevertheless, HPgV-1 RNA load increased steadily from baseline (median, 0; IQR 0–4.78 log_{10} copies/mL) over the study period (peak level, 4.04 log_{10} copies/mL; IQR, 0–5.35 reached by day +120) (Figure 1, Table 2). No demographic or clinical variables were associated with the rate of TTV, TAV, or HPgV-1 detection in our cohort (not shown).

3.2 | Monitoring of Torque teno virus, total Anelloviridae and Human Pegivirus-1 plasma load for the prediction of infection events

First, we aimed to investigate whether TTV, TAV, and HPgV-1 viral loads measured at different time points before or after allo-HSCT differed across patients who either did or did not develop certain infection events. Since *Anelloviridae* and HPgV-1 burden is presumed to be modulated by virus-specific T cells, only infection events occurring in the study population within the study period likely associated with impaired T-cell responses were considered in the analyses described below; namely, pneumonia (occurring a median of 44 days; range 4–144 after transplantation) due to SARS-CoV-2 ($n = 3$), *Pneumocytis jirovecii* ($n = 1$), or CMV ($n = 1$), CMV DNAemia (detected a median of 47 days after transplantation; range, −8 to 49) ($n = 36$), and BK virus-associated Hemorrhagic cystitis (BKPyV-HC) ($n = 18$), diagnosed a median 21 days after transplantation (range, 8–82). A total of 40 patients (53%) developed one or more infectious events during the first 180 days after allo-HSCT; specifically, 32 (43%) patients had an episode of infection during the first 30 days, 6 (8%) between days +31 and +60 and 6 (8%) between days +61 and +90.

Due to the above distribution of infectious events across time, we focused on assessing whether TTV, TAV, and HPgV-1 loads quantitated either before transplantation at baseline or by day +30 were associated with the occurrence of overall infection developing after these time points. As shown in Table 3, this was not the case. Likewise, a lack of association was observed when viral loads only from patients testing positive by the respective PCR assay were considered for analyses (Supporting Information: Table 2). Moreover, from a qualitative standpoint, testing positive by for any of the above viruses before transplantation at baseline and at day +30 was not associated with the occurrence of infectious events (*p* values ranging from 0.12 to 0.87).

A sufficient number of early BKPyV-HC ($n = 11$) and CMV DNAemia episodes ($n = 36$), that is developing within the first 30 days after transplantation, were registered to independently assess the potential association between TTV, TAV, and HPgV-1 loads measured either before transplantation or at baseline and occurrence of these clinical events within this time frame. While no significant differences between TTV, TAV, and HPgV-1 loads were observed across patients either developing CMV DNAemia or not (Supporting Information: Table 3), both pretransplant and baseline TTV DNA levels were significantly higher in patients who subsequently developed BKPyV-HC compared to that in whom the disease was not diagnosed (median 5.26 $\log_{10}$ copies/mL [IQR, 4.60–7.08] vs. 3.85 $\log_{10}$ copies/mL [IQR, 0–5.19]; $p < 0.01$ for pretransplant and median 5.89 $\log_{10}$ copies/mL [IQR, 4.92–6.86] vs. 3.45 $\log_{10}$ copies/mL [IQR, 0–5.29] $p < 0.01$ for baseline) (Figure 2A). ROC analysis showed that both preconditioning and baseline TTV viral loads had similar predictive ability for early BKPyV-HC; in effect, preconditioning TTV viral loads greater than 4.40 $\log_{10}$ copies/mL had a sensitivity of 89% (95% CI, 54%–99%), specificity of 63% (95% CI, 48%–76%), positive predictive value (PPV) of 33% and negative predictive value (NPV) of 96% (AUC 0.78; $p < 0.001$). Likewise, baseline TTV viral load greater than 4.58 $\log_{10}$ had a sensitivity of 100% (95% CI, 62%–100%), specificity of 65% (95% CI, 49%–78%), PPV of 38% and NPV of 100% (AUC 0.84; $p < 0.001$). When only patients testing positive for TTV were considered for analyses, a trend towards higher pretransplant viral loads in patients developing BKPyV-HC was observed (median, 5.26 $\log_{10}$ copies/mL; IQR, 4.68–6.88, vs. median, 4.44 $\log_{10}$ copies/mL; IQR, 3.71–5.39; $p = 0.08$). In turn, patients developing BKPyV-HC had significantly higher ($p = 0.02$) TTV DNA loads at baseline than those who did not (median, 5.89 log10 copies/mL; IQR, 5.07–6.65 vs. median, 4.58 log10 copies/mL; IQR, 3.39–5.59). In these patients, baseline TTV viral loads greater than 5.37 $\log_{10}$ copies/mL displayed a sensitivity of 75% (95% CI, 44.9%–100%), specificity of 78.4% (95% CI, 63.4%–93.3%), PPV of 42% (95% CI, 21.4–67.4) and NPV of 93.5% (95% CI, 79.3–98.2) (AUC, 0.78; $p = 0.018$). In turn, testing positive or negative for TTV before transplantation, at baseline or at day +30 was not associated with the subsequent occurrence of BKPyV-HC (*p* values, 1.0, 0.24, 0.14, respectively).

Importantly, frequency analyses revealed that receipt of an haploidentical or HLA-mismatched allograft was more common

TABLE 3 Plasma Torque teno virus DNA, total *Anelloviridae* DNA, and Human Pegivirus 1 RNA loads measured before, at the time of allogeneic hematopoietic stem cell transplantation or by day +30 in patients with or without infections (one or more) and acute graft vs. host disease (all grades) occurring within the first 180 days after transplantation.

Virological parameter	Infectious events[a]			Acute graft vs. host disease[b]		
TTV (median $\log_{10}$ copies/mL [IQR]	**Yes**	**No**	***p* Value**	**Yes**	**No**	***p* Value**
Pretransplant	4.41 [2.95–5.52]	3.98 [0–5.19]	0.41	4.00 [0–5.45]	4.29 [3.24–5.30]	0.72
Baseline	4.52 [0.77–5.79]	4.05 [3.02–5.55]	0.93	4.31 [3.18-6.45]	4.41 [0–5.60]	0.58
+30	3.80 [2.53–4.51]	3.14 [0–4.58]	0.46	4.53 [3.63–6.04]	2.66 [0–4.37]	***0.02***
TAV (median $\log_{10}$ copies/mL [IQR]	**Yes**	**No**	***p* Value**	**Yes**	**No**	***p* Value**
Pretransplant	0 [0–4.08]	0 [0–3.08]	0.80	0 [0–3.37]	0 [0–3.33]	0.65
Baseline	0 [0–2.57]	0 [0–2.69]	0.64	0 [0–3.31]	0 [0–2.32]	0.21
+30	0 [0–4.32]	0 [0–0]	0.21	0 [0–0]	0 [0–0]	0.47
HPgV-1 (median $\log_{10}$ copies/mL [IQR])	**Yes**	**No**	***p* Value**	**Yes**	**No**	***p* Value**
Pretransplant	2.08 [0–4.33]	0 [0–5.49]	0.71	0 [0–3.26]	3.48 [0–5.48]	***0.03***
Baseline	0 [0–4.21]	0 [0–3.54]	0.61	0 [0–3.61]	0 [0–4.25]	0.49
+30	2.29 [0–4.50]	2.17 [0–4.30]	0.92	2.72 [0–4.59]	0 [0–4.32]	0.69

Abbreviations: HPgV-1, Human Pegivirus-1; TAV, total anelloviridae; TTV, Torque teno virus.

[a]A total of 39 patients had one or more infectious events, including pneumonia due to SARS-CoV-2 ($n = 3$), *Pneumocytis jirovecii* ($n = 1$), or cytomegalovirus ($n = 1$), cytomegalovirus DNAemia ($n = 36$), and BK virus-associated hemorrhagic cystitis ($n = 18$).

[b]A total of 29 patients developed acute graft vs. host disease (any grade).

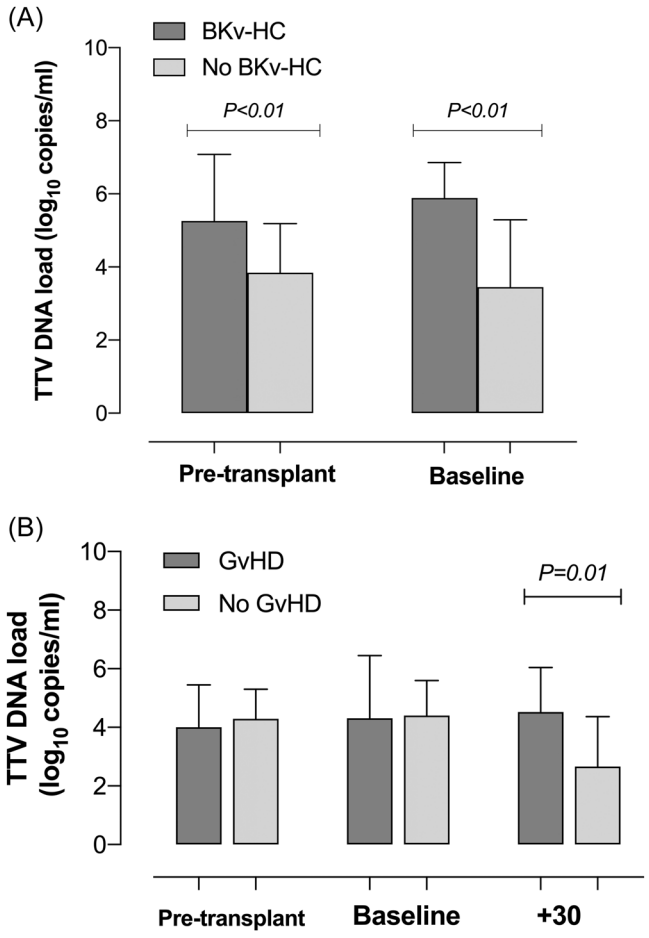

FIGURE 2 Association of plasma Torque teno virus DNA loads and subsequent occurrence of BK polyomavirus-associated haemorrhagic cystitis (A) or acute graft versus host disease of any grade (B). Significant *p* values are shown.

among patients who developed early BKPyV-HC (82% vs. 39%; $p = 0.03$). In contrast, both preconditioning and baseline TAV or and HPgV-1 loads were comparable in both comparison groups.

3.3 | Monitoring of Torque teno virus, total Anelloviridae and Human Pegivirus-1 plasma load for the prediction of acute graft versus host disease

A total of 29 patients (39%) developed aGvHD at a median of 29 days after transplantation (range, 6–133), of whom 18 had grades II–IV disease and received corticosteroids at high doses (>1 mg/kg/day). Plasma TTV, TAV, and HPgV-1 loads measured before transplantation, at baseline or by day +30 in patients with or without aGvHD (any grade) are shown in Table 2. Overall, median TTV DNA load determined by day +30 was significantly higher (median 4.53 $\log_{10}$ copies/mL vs. 2.66 $\log_{10}$ copies/mL; $p = 0.02$) in patients who went on to develop aGvHD than in those who did not (Figure 2B). A similar observation was made when only grade II–IV aGvHD were considered (median 5.81 $\log_{10}$ copies/mL vs. median 2.79 $\log_{10}$ copies/mL; $p < 0.001$). ROC analysis (AUC = 0.73; $p = 0.02$) showed that TTV viral loads above 3.38 $\log_{10}$ copies/mL by day +30 could predict the occurrence of subsequent aGvHD with 90% sensitivity (95% CI, 59.6–98.2), 57% specificity (95% CI, 43.3–69.0), 28% PPV (95% CI,.15.6–45.4), and 97% NPV (95% CI; 83.8–99.4). Likewise, TTV viral loads above 5.07 $\log_{10}$ predicted the occurrence of aGvHD grades II-IV with 83% sensitivity (95% CI; 43.6–97.0), 91% specificity (95% CI,81.1–96.2), 50% PPV (95% CI, 23.7–76.3), and 98% NPV (95% CI,90.1–99.7); (AUC = 0.88; $p = 0.002$).

Among patients testing positive for TTV DNA by day +30, TTV DNA loads were significantly higher ($p = 0.017$) in those developing aGvHD grades II–IV (median, 5.81 $\log_{10}$ copies/mL; IQR, 5.16–6.34 vs. median, 4.11 $\log_{10}$ copies/mL; IQR, 3.41–4.71), while viral loads were comparable ($p = 0.28$) across patients with or without all grade aGvHD. In this sense, TTV viral loads above 5.11 $\log_{10}$ copies/mL predicted the occurrence of aGvHD grades II–IV with 83% sensitivity (95% CI.43.6–97.0), 85.0 specificity (95% CI.69.9.1–93.6), 50% PPV (95% CI.23.7–76.3), and 96.6% NPV % 95% CI.83.3–99.4); (AUC, 0.80; $p = 0.019$).

Finally, the detection rate (any level) of TTV DNA by day +30 was not different across patients with or without all grade aGvHD (17/25 vs. 23/39; $p = 0.46$) or aGvHD grades II–IV (11/15 vs. 29/49; $p = 0.38$). Interestingly, as shown in Table 3, overall, pretransplant but not baseline HPgV-1 loads were significantly higher ($p = 0.03$) in patients who had not aGvHD than in those who did so (median, 3.48 $\log_{10}$ copies/mL vs. undetectable); this observation stand when only grade II–IV episodes were considered for analyses (median, 2.98 $\log_{10}$ copies/mL vs. undetectable; $p = 0.03$) or when only patients testing positive for HPgV-1 by PCR at this time point were included in the analysis (median, 5.45 $\log_{10}$ copies/mL; IQR 4.19–5.84 vs. median, 3–71; IQR, 2.87–4.20; $p = 0.003$). In this context, notably, detectability of HPgV-1 before transplantation was associated with a lower incidence of grades II–IV aGvHD (detectable in 24 out of 44 patients without the disease and in 1 out of 8 patients with the disease; $p = 0.05$).

4 | DISCUSSION

Here, we further characterized the kinetics of TTV, TAV, and HPgV-1 plasma load in allo-HSCT and investigated whether viral burdens estimated at different time points after transplant could predict the occurrence of certain infectious events that developed in our patients and are likely associated with deficient T-cell responses, including pneumonia due to SARS-CoV-2, *P. jirovecii*, or CMV, BKPyV-HC and CMV DNAemia and severe aGvHD (or its treatment with corticosteroids). To quantify TAV DNA we used an "in house" RT-PCR that returned lower viral loads than that provided by our TTV RT-PCR and was less sensitive,[14] despite being able to target *Anelloviridae* other than TTV. This extent was previously shown by our group in a cohort of SOT recipients.[29]

The kinetics of TTV DNA load followed a previously characterized pattern,[8,9] with lowest levels measured around the time of

engraftment and peak levels quantified by day +90. Since TTV and TAV DNA levels correlated well (Rho, 0.71), not surprisingly the kinetics of TAV DNAemia mirrored that of TTV DNAemia. HPgV 1 RNA was detected (at least in once) in around 66% of patients, a figure slightly higher than previously reported (range, 14%–61%).[17–23] Although speculative, differences between analytical performances of RT-PCR used in these studies or history of blood components replacement across patients in the cohorts, among other factors may account for this finding. Notably, although the rate of HPgV-1 detection remained relatively stable throughout the study period, de novo acquisition HPgV-1 following transplantation may have taken place in some patients, those testing negative in the preconditioning sample and positive in one or more posttransplant specimens. Nevertheless, we cannot rule out that these patients were already infected before transplantation and displayed HPgV-1 RNA loads below the limit of the detection of the assay. Likewise, our data suggested that a number of patients might have acquired *Anelloviridae* infection in the posttransplant period. Interestingly, the dynamics of HPgV-1 RNA load was found to follow a similar trend compared to TTV DNA or TAV DNA loads, although peak levels were measured later (by day +120). This finding can be interpreted as further indicating the lymphotropic nature of HPgV-1 and the potential relevance of T-cell dependent mechanisms in the ultimate control of virus replication.

In our view, the most relevant observations of the current study are as follows. First, while neither TTV, TAV, nor HPgV 1 levels measured at different time points appeared to anticipate the occurrence of subsequent infectious events considered collectively, we found that, pretransplant and baseline TTV DNA loads predicted the risk of BKPyV-HC developing early after transplantation (within the first 30 days), irrespective of whether all patients or only those testing positive by PCR at these time points were considered for analyses. Specifically, overall, a TTV viral load cut-off ≥4.40 $\log_{10}$ (pretransplant) and ≥4.58 $\log_{10}$ (baseline) copies/mL displayed a high sensitivity (89%) and NPV (96%) for predicting BKPyV-HC. This suggested that TTV DNA load may behave as a surrogate marker for immunosuppression driven by either the underlying hematological disease, its treatment before transplantation the conditioning regimen used or all together. Strikingly, a quite similar cut-off (≥5.01 $\log_{10}$ copies/mL) measured by day +30 was predictive of the occurrence of BKPyV-HC after kidney SOT.[30] Other studies conducted in the kidney SOT setting also found an association between high TTV DNA loads and high BKV DNAemia.[31] On the other hand, no significant differences between TTV, TAV, and HPgV-1 loads were observed across patients either developing CMV DNAemia (at any level) or not, which concurred with previous findings from our group (relative to TTV DNA load).[9]

Second, in our setting, high TTV DNA viral loads measured by day +30 (after engraftment) were associated with the subsequent occurrence of aGvHD (all grades or grade II–IV), regardless either all tested patients or only those testing positive by PCR were considered for analyses. Noticeably, TTV viral loads above 5.07 $\log_{10}$ predicted the occurrence of aGvHD grades II–IV with high sensitivity (83%) and very high NPV (98%). This observation could be explained on the basis of the immune-mediated pathogenetic nature of aGvHD and the fact that TTV DNA load at around the time of engraftment seems to be more reflective of immune reconstitution, rather than immunosuppression.[8–10] In line with our finding, Gilles et al.[11] reported high TTV DNA by day +30 in patients who developed aGvHD during the first 100 days after transplantation. A direct link between TTV DNA levels and aGvHD has also be noticed in other studies.[13,23] In contrast, Schmitz and colleagues were unable to predict the occurrence of immune-related clinical complications on the basis of TTV DNA monitoring.[12] Differences in clinical and demographic characteristics of patients in these cohorts may account for the observed discrepancies.

Third, HPgV-1 infection has been associated with immunomodulatory effects and benefitial clinical outcomes in HIV and Ebola infected individuals.[16] In the allo-HSCT setting, HPgV-1-viremic recipients were found to display an impaired NK cell, but not T cell, immune-reconstitution compared with HPgV-1-non-viremic patients in a previous study,[24] thus suggesting an immunomodulatory role for HPgV-1; yet, to our knowledge, a few studies conducted in the allo-HSCT setting have investigated whether an association exists between HPgV-1 and any clinical outcome including infection, aGvHD or survival.[19–21] Here, we found pre-transplant HPgV-1 RNA loads to be significantly lower in patients who had aGvHD (all grades or grade II–IV) compared to those who did not. A similar observation was made when only patients testing positive at this time point were included. Furthermore, patients testing positive for HPgV-1 RNA before conditioning had a lower rate of aGvHD (all grades or grades II–IV) compared to patients testing negative. In line with this latter observation, Vu et al.[21] reported a trend ($p = 0.19$) towards a lower incidence of grades II–IV aGvHD in patients testing positive for HPgV-1 at the time of transplantation compared to that in patients who did not. Nevertheless, since virus loads measured at baseline or at any time after transplantation were not associated with the subsequent risk of developing aGvHD the interpretation of our finding is not straightforward; although it could be argued that preconditioning HPgV-1 RNA load may be somehow reflective of the impact of the underlying disease and its treatment on the state of immunocompetence, which may also influence the risk of aGvHD, at this point, and given the small sample size of our study, we cannot rule out the possibility that this is merely artefactual.

The main limitation of the current study is undoubtedly its limited sample size that prevented us from analyzing the impact of factors that may associate with clinical outcomes and blood viral burden, including the nature of the underlying disease, type of allo-HSCT, or the immunosuppressive regimen used or a potential "dose-dependent" effect of TTV DNA load on the risk of BKPyV-HC. In addition, a few patients ($n = 15$) had all set of plasma specimens, which impeded a more precise characterization of the kinetics of viral loads for these viruses. This could have resulted in a miscategorization of some patients, specially those with a single positive PCR specimen during follow-up, which were considered as infected by the corresponding virus (i.e., patient 23 for HPgV-1). Moreover, due to its

monocentric nature, it is uncertain whether our findings might be generalizable to other centers. In conclusion, pretransplant or baseline TTV DNA and HPgV-1 RNA plasma levels may be ancillary to identify allo-HSCT recipients at increased risk of BKPyV-HC or aGvHD. Further studies are warranted to prove or refute this assumption.

AUTHOR CONTRIBUTIONS

Lorena Forqué and Eliseo Albert performed the experiments and analyzed the data. José Luis Piñana, Ariadna Pérez, Rafael Hernani, and Carlos Solano contributed to data curation and analysis. David Navarro and Estela Giménez designed the study, analyzed the data, and wrote the manuscript. All authors approved the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the statements in this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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