# Human cytomegalovirus virion-associated mRNA as a marker of productive infection in immunocompromised patients.

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**Abstract**

Human Cytomegalovirus (HCMV) transcripts (including UL21.5 mRNA) have been found to be packaged in virions and their detection in plasma may indicate the presence of infectious viral particles. Objective of this study was to verify whether UL21.5 mRNA detected in the plasma was indeed encapsulated in viral particles, representing an indirect marker of active replication. To distinguish between virion-packaged and free-floating RNA, plasma samples from 22 immunocompromised patients were tested before and after ribonuclease (RNAse) digestion. UL21.5 mRNA was detected 1-2 weeks prior to preemptive therapy administration in 20 episodes (from 18 patients) of clinically significant DNAemia, while it was undetectable in three of the four patients with transient, self-resolving DNAemia. After RNAse digestion, UL21.5 mRNA was still detectable, with a median reduction of 0.1 (IQ range 0-0.3) Log $_{10}$. Concentrations of UL21.5 mRNA in plasma correlated significantly with HCMV DNA in whole blood or plasma (R=0.67), and 75% of samples positive for UL21.5 mRNA had HCMV DNA concentrations above 10 $^{4}$ copies/ml blood or 10 $^{3}$ copies/ml plasma. Moreover, UL21.5 mRNA was positive in patients who developed HCMV infection resistant to letermovir or maribavir, whereas it was undetectable in plasma of patients with transient self-resolving DNAemia blips during letermovir prophylaxis (not associated with drug-resistance). HCMV UL21.5 mRNA in plasma is virion-associated and represents a marker for productive HCMV infection. The determination of UL21.5 mRNA could improve current strategies for the management of HCMV infection in immunocompromised patients.

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**Running head:** Virion-associated CMV mRNA in plasma

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**Conflict of interest disclosure**

The authors declare no conflict of interest

**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.

**Patient consent statement**

*Informed consent was not required according to the national regulation for retrospective studies in research hospitals.*

## ABSTRACT

*Human Cytomegalovirus (HCMV) transcripts (including UL21.5 mRNA) have been found to be packaged in virions and their detection in plasma may indicate the presence of infectious viral particles. Objective of this study was to verify whether UL21.5 mRNA detected in the plasma was indeed encapsulated in viral particles, representing an indirect marker of active replication. To distinguish between virion-packaged and free-floating RNA, plasma samples from 22 immunocompromised patients were tested before and after ribonuclease (RNAse) digestion. UL21.5 mRNA was detected 1-2 weeks prior to preemptive therapy administration in 20 episodes (from 18 patients) of clinically significant DNAemia, while it was undetectable in three of the four patients with transient, self-resolving DNAemia. After RNAse digestion, UL21.5 mRNA was still detectable, with a median reduction of 0.1 (IQ range 0-0.3) $Log_{10}$. Concentrations of UL21.5 mRNA in plasma correlated significantly with HCMV DNA in whole blood or plasma (R=0.67), and 75% of samples positive for UL21.5 mRNA had HCMV DNA concentrations above $10^4$ copies/ml blood or $10^3$ copies/ml plasma. Moreover, UL21.5 mRNA was positive in patients who developed HCMV infection resistant to letermovir or maribavir, whereas it was undetectable in plasma of patients with transient self-resolving DNAemia blips during letermovir prophylaxis (not associated with drug-resistance). HCMV UL21.5 mRNA in plasma is virion-associated and represents a marker for productive HCMV infection. The determination of UL21.5 mRNA could improve current strategies for the management of HCMV infection in immunocompromised patients.*

**Key words:** human cytomegalovirus; mRNA; virion; transplant recipients; immunocompromised patients.

## 1 INTRODUCTION

*Human Cytomegalovirus (HCMV) is still one of the most concerning infection in transplant recipients. Prevention of clinically relevant HCMV infections depends on HCMV surveillance during the first year after*

*transplantation. Currently, quantification of HCMV DNA in blood samples (HCMV-DNAemia) represents the gold standard for identifying active viral replication, guiding antiviral therapy to prevent HCMV-related disease and monitoring response to drugs targeting HCMV DNA polymerase. While ganciclovir (GCV), valganciclovir (VGCV) and foscarnet (PFA), which are widely used for HCMV therapy, target the viral DNA polymerase (UL54), new antiviral drugs such as letermovir (LTV) and maribavir (MBV) have different targets and mechanisms of action. LTV inhibits the cleavage of viral DNA concatamers and their packaging into virions by targeting the HCMV terminase complex, while MBV interferes with viral pUL97 kinase activity, morphogenesis and nuclear egress of nascent viral particles [1]. In particular, LTV has been proven in a phase-III clinical trial to reduce the risk of clinically significant HCMV infection [2]. Thanks to its safety and lack of myelotoxicity, LTV is approved for prophylaxis in adult haematopoietic stem cell transplant recipients (HSCTR).*

*Real-life experience in a cohort of HSCTR revealed that HCMV DNA can be detected in whole blood and plasma samples during prophylaxis even in the absence of productive viral replication [3]. This is probably related to the different mechanisms of action of LTV, which, by inhibiting the later stages of virion assembly, allow the synthesis of DNA (abortive infection) that can be detected by standard molecular tests in the absence of infectious virus production (DNAemia blips).*

*Additional methods, including the HCMV viremia (detection of HCMV-infected cells after rapid isolation of HCMV using fibroblasts) and quantification of HCMV DNA in plasma after digestion with deoxyribonuclease (DNAse) (to identify DNA packaged in virions), have been proposed to identify productive viral replication [3,4]. However, the HCMV viraemia test must be performed within 24 hours of sample collection and is less sensitive than molecular assays. On the other hand, the DNase test is a time-consuming and poorly standardized test that requires the use of DNAse I in the sample before extraction to distinguish free-floating DNA from the genome packaged in virions [3,4].*

*Alternative and simpler methods to identify active HCMV replication could be a valuable improvement in monitoring transplanted patients and guiding therapy, particularly in patients receiving LTV. In the past years, the determination of viral transcripts in whole blood for monitoring HCMV replication has been proposed [5-7]. Recently, an assay targeting virion-associated UL21.5 mRNA, a late transcript that is highly expressed and packaged into virions during lytic infection [8], has been developed.*

*The primary objective of this study was to verify that UL21.5 mRNA detected in plasma is actually included into viral particles and could represent an indirect marker for active replication. For this aim, retrospectively collected plasma samples from immunocompromised patients were tested for HCMV UL21.5 mRNA before and after ribonuclease (RNAse) digestion (RNAse protection assay) to distinguish between virion-packaged and free-floating naked RNA.*

## 2 MATHERIALS AND METHODS

### *2.1 Study design*

*In this study, plasma (stored frozen at -80°C within 48 hours after collection, without any additional RNA preservative) from residual blood samples of 22 immunocompromised patients experiencing HCMV DNAemia episodes in whole blood (WB) were retrospectively analysed. HCMV infection was monitored at the Fondazione IRCCS Policlinico San Matteo, Pavia, by weekly determination of WB DNAemia. Pre-emptive antiviral therapy with GCV or VGCV was administered in 20 episodes of WB DNAemia of 18 patients (two of whom had two different episodes of HCMV DNAemia), while four patients had a transient, self-resolving episode of WB DNAemia that did not require pre-emptive therapy (Table 1).*

*Plasma (PL) samples were retrospectively analysed for HCMV UL21.5 mRNA and PL DNAemia. The primary aim of this study was to verify whether UL21.5 mRNA is detectable in plasma during WB HCMV DNAemia episodes and whether the detected RNA is packaged in virions or released as free-floating RNA by degradation of infected cells.*

*In addition, four plasma samples from episodes of breakthrough DNAemia occurring due to antiviral drug*

*resistance in patients receiving MBV (two samples from a kidney transplant recipient -KTR- and one from a HSCTR) or LTV (one HSCTR) were tested for UL21.5 mRNA, as well as samples from 5 patients (one KTR and four HSCTR) developing self-resolving transient DNAemia "blips" (not associated with antiviral drug resistance) during LTV prophylaxis.*

*Informed consent was not required according to the national regulation for retrospective studies in research hospitals.*

*2.2 HCMV DNA detection*

For WB-DNAemia detection in routine diagnostic procedures, viral DNA was extracted from 200 µl of whole blood using QIAsymphony® DSP DNA Mini kit (Qiagen, Heidelberg, Germany) on the Qiasymphony platform (Qiagen, Heidelberg, Germany). Real time-PCR master mix was prepared using QuantiFast Pathogen PCR+IC KIT (Qiagen, Heidelberg, Germany) according to the manufacturer's instructions. The sequences of the primers and probes used were previously described [9,10]. Reactions were performed on the Rotor-Gene Q thermocycler (Qiagen, Heidelberg, Germany) with the following thermal protocol: 95°C for 5 min then 45 cycles at 95°C for 15sec and 60°C for 30sec.

For PL DNAemia, 200µl of plasma was extracted using QIAamp® DSP Virus Spin kit. Real PCR assays were performed using TaqMan® Universal PCR MasterMix (Applied Biosystems, USA) and the same primers and probes set. Reactions were performed on 7300 Real time PCR system thermocycler with the following thermal protocol: 50°C for 2min, 95°C for 10min, 50 cycles at 95°C for 15sec and 60°C for 1min. To detect virion-associated PL DNAemia, plasma was treated with DNAse before DNA extraction as previously reported [3]

*2.3 HCMV UL21.5 mRNA detection*

To evaluate the presence of UL21.5 mRNA, nucleic acids were extracted from 600µl of plasma using ELITe InGenius® SP1000. Realtime RT-PCR was performed using CMV RNA ELITe MGB® kit (kindly provided by ELITech Group) targeting the UL21.5 gene, according to the manufacturer's instructions. Nucleic acids extraction, UL21.5 cDNA synthesis and amplification and results interpretation were performed on the ELITe InGenius® platform (ELITech Group, USA). To obtain a reliable quantification, standard samples (concentrated $10^2$-$10^4$ copies/reaction) were tested once every 60 days, following the manufacturer's instructions. Results were expressed in copies/ml. Samples in which UL21.5 mRNA was detected but at levels below the quantification limit (30 copies/ml) were assigned an arbitrary value of 10 copies/ml. Negative samples were reported as "undetectable".

*2.4 RNAse digestion on plasma samples (RNAse protection assay)*

To develop the digestion protocol, 100µl of Rhinovirus (HRV) RNA in Tris-Borate EDTA solution (8,5x$10^8$ copies/ml) and its scaling dilutions were treated with 2µl of RNase ONE$^{TM}$Ribonuclease for two and a half hours as reported by Wu et al, 2012 [11]. HRV RNA quantification after RNAse digestion was performed with an in-house assay targeting 5'-UTR region.To investigate if the total amount of HCMV UL21.5 mRNA detected was packaged in virions or naked, RNAse digestion was performed on 600 µl plasma samples. After RNAse digestion, HCMV UL21.5 mRNA detection was performed on the ELITe InGenius® platform (ELITechGroup, USA) as previously described. To evaluate the actual effectiveness of RNAse digestion in plasma, the first 14 samples were added with 15µl of HRV RNA solution (6,7x$10^6$ copies/ml). After digestion, HRV RNA presence was investigated as described above.

**2.5 HCMV DNA and UL21.5 mRNA quantification in purified virus preparations**

**Τωο διφφερεντ τψπες οφ ςελλ ςυλτυρες ωερε υσεδ φορ ιρυς στραιν προπαγατιον: διπλοιδ ΗΕΛΦ (ηυμαν εμβρψονις λυνγ φιβροβλαστ) ανδ α ςελλ λινε οφ ηυμαν ρετιναλ πιγμεντεδ επιτηελιαλ ςελλς (ΑΡΠΕ-19, ΑΤ"" "ΡΛ-2302, Μανασσας, "Α, ΥΣΑ). ΗΕΛΦ ωερε δεριεδ φρομ α ςελλ στραιν δεελοπεδ ιν ουρ λαβορατορψ ιν 1980 ανδ υσεδ ατ πασσαγες 20–30. ΑΔ169 α ηιγηλψ πασσαγεδ λαβορατορψ-αδαπτεδ στραιν ωας ινοςυλατεδ**

**οντο ΗΕΛΦ ςελλς ανδ τηε Η"Μ" ισολατε "Ρ1814, ωηιςη ωας οριγιναλλψ ρεςοερεδ φρομ ςεριςαλ σεςρετιονς, οντο ΑΡΠΕ-19 ςελλς υντιλ α ςελλ-φρεε ιρυς στοςκ ωιτη α συφφιςιεντ ινφεςτιους τιτερ (2ξ10$^6$ ανδ 4ξ10$^6$, ρεσπεςτιελψ) ωας οβταινεδ ιν τηε ςελλ-ςυλτυρε συπερναταντ. Τηε ινφεςτεδ ςελλ συπερναταντ ωας τηεν φιλτερεδ ωιτη α στεριλε σψρινγε υσινγ 0.45 μμ φιλτερ ("ΩΡ Ιντερνατιοναλ Σρλ, Ραδνορ, ΠΑ, ΥΣΑ) ιν ορδερ το ελιμινατε ςελλυλαρ δεβρις. ΥΛ21.5 μPNA χυαντιφιςατιον ανδ PNAσε προτεστιον ασσαψ ωερε περφορμεδ ας δεσςριβεδ αβοε.**

### 2.6 Quantification of leukocytes carrying infectious virus

The number of leukocytes carrying the infectious virus was quantified by inoculating 2x10$^5$ leukocytes from peripheral blood onto cell cultures of human embryonic lung fibroblasts using the shell-vial technique. The number of fibroblast nuclei positive for the HCMV immediate-early antigen p72 was then counted 16 to 24 hours after infection [12]

### 2.7 Statistical analysis

Quantitative variables are presented as median and interquartile (IQ) range. In order to quantify the kinetics of HCMV DNAemia and UL21.5 mRNA, data were normalized by considering 100% the peak value of viral load detected by different assays the day of starting therapy (day 0) and then referring to it as percentages all values detected by the relevant assay either prior to or after onset of therapy. The median values detected in 7-day intervals before or after starting of therapy were calculated. Kaplan-Meier curves and log-rank test were used to compare the time to clearance of DNAemia and UL21.5 mRNA. The Pearson's correlation analysis was performed between UL21.5 mRNA and the other virological parameters, using $Log_{10}$ values of HCMV DNA and UL21.5 mRNA load.

## 3 RESULTS

### 3.1 Detection of UL21.5 mRNA during DNAemia episodes.

Peak levels of WB and PL DNAemia in patients requiring pre-emptive therapy or showing self-resolving transient DNAemia are shown in Table 1 and Figure 1B. UL21.5 mRNA was detected in plasma 1-2 weeks before administration of pre-emptive therapy in all the 20 episodes of clinically significant DNAemia (Table 1 and Figure 1A), whereas it was undetectable in 3 of the 4 patients with transient self-resolving DNAemia (the only positive patient showed very low UL21.5 mRNA level below the limit of quantification). The median kinetics of WB and PL DNAemia and of UL21.5 mRNA (in plasma) in the 20 episodes of clinically significant DNAemia is shown in Fig 1B. UL21.5 mRNA was detected about two weeks after WB DNAemia appearance, and, during pre-emptive therapy, it was cleared earlier than WB DNAmia. After start of treatment, median time to UL21.5 mRNA clearance was 16 days, whereas median time to WB DNAemia and PL DNAemia was 36 days (Fig. 1C).

### 3.2 UL21.5 mRNA in plasma is mostly packaged in virions

We tested UL21.5 mRNA both in purified virus preparations and in plasma of infected patients before or after RNAse digestion (RNAse protection assay) to verify whether it is free-floating in plasma (and therefore undetectable after digestion) or packaged in virions (and therefore resistant to RNAse digestion). Plasma amples with UL21.5 mRNA levels above the quantification limit (n=28) were included in this analysis. To verify the efficacy of the RNA digestion treatment, different concentrations of rhinovirus RNA solution (from 10$^9$ to 10$^4$ copies/ml) were treated with the same protocol that was subsequently applied to the plasma samples, resulting in complete degradation of the RNA at all the concentrations tested (Figure 2A).

After RNAs digestion of purified virus preparations of AD169 and VR1814, UL21.5mRNA was still detectable with a reduction of 0 and 0.3 $Log_{10}$, respectively (Figure 2B).

Similarly, after RNAse digestion of the plasma, UL21.5 mRNA was still detectable in all samples, with a median reduction of 0.1 (IQ range 0-0.3) $Log_{10}$ (Figure 2C). This indicates that the UL21.5 mRNA detected

in plasma is mainly packaged in virions. As control, in the first 14 samples tested $10^6$copies/ml of rhinovirus RNA were added to plasma. After RNAse treatment, rhinovirus RNA was undetectable in all samples.

### 3.3 Correlation between UL21.5 mRNA in plasma and HCMV DNAemia or viremia

There was a significant correlation between plasma UL21.5 mRNA levels and WB DNAemia or PL DNAemia (Fig. 3A and B). The correlation was similar to WB DNAemia or PL DNAemia (R=0.67). The viremia (i.e. the number of leukocytes carrying infectious virus) was determined in 24 samples. The correlation between UL21.5 mRNA in plasma and viremia was weaker (R=0.44; Fig. 3C). In particular, UL21.5 mRNA was also detectable in samples with undetectable viremia.

Considering cut-off values of 104 copies/ml for WB DNAemia or 103 copies/ml for PL DNAemia, 75% of samples positive for UL21.5 mRNA had WB and PL DNAemia values above these cut-off values (Fig. 4). Conversely, only a few samples that were negative for UL21.5 mRNA had DNAemia values above 104 or 103 copies/ml WB or PL (10% and 25% of samples, respectively).

### 3.4 UL21.5 mRNA in plasma of patients developing drug resistance or self-resolving DNAemia blips during LTV/MBV treatment.

UL21.5 mRNA was tested in four plasma samples collected from patients (KTR and HSCTR) developing drug-resistant HCMV infection during LTV prophylaxis or MBV treatment. The mRNA was detected in the four samples analysed at levels between 60 and 586 copies/ml. In parallel, plasma samples from four HSCTR and one KTR showing transient self-resolving DNAemia blips during LTV prophylaxis were also analysed. UL21.5 mRNA was undetectable in all the 5 samples tested (Fig 5).

## 4 DISCUSSION

The results of the present study show that human cytomegalovirus UL21.5 mRNA is detectable in plasma during clinically significant episodes of HCMV DNAemia in immunocompromised patients. The mRNA detected in plasma is mostly virion-encapsulated, and its levels correlate with those of HCMV DNAemia. UL21.5 mRNA is absent in plasma of patients showing transient self-resolving DNAemia blips during LTV prophylaxis (i.e. abortive infection), whereas it is detectable in patients with HCMV DNAemia because of HCMV infection resistant to LTV or MBV (i.e. productive virus replication).

HCMV DNAemia monitoring have been used for more than 20 years to monitor HCMV infection in immunocompromised patients. Detection of HCMV DNA is generally considered a direct marker of active viral replication [13,14] and is widely used to guide pre-emptive therapy of HCMV infection in transplant recipients [15,16] and to monitor the efficacy of antiviral treatment [14,17].

However, the novel antiviral drugs recently introduced, such as LTV and MBV inhibit the production of infectious viruses without interfering with HCMV DNA synthesis, which accumulates in infected cells as non-infectious DNA or immature viral particles [18-20]. Consequently, viral DNA not associated with infectious virions can be released in the bloodstream by infected cells undergoing lysis. Therefore, the presence of HCMV DNA in blood during antiviral treatment with these antiviral agents should be considered with caution [4], and alternative markers of productive HCMV infection should be investigated.

The detection of infectious viruses in blood through its isolation from virus-carrying leukocytes (i.e. viremia) would be the ideal parameter for the identification of productive viral infection. However, the assay for viremia quantification is not sensitive enough for the early diagnosis of active infection episodes.

In order to discriminate between the presence of free-floating DNA or virion-associated DNA, pre-treatment of plasma with DNAse has been proposed [3,4]. Free-floating DNA is disrupted by DNAse treatment, while virion-associated DNA remains detectable. Thus, detection of HCMV DNA in plasma after DNAse treatment may represent a surrogate marker of the presence of infectious HCMV in blood.

In addition to genomic DNA, mRNA molecules are also packaged into virions, specifically into the tegument compart. Originally, five transcripts were detected in the virions, UL21.5, UL 106–109, TRL/IRL 2–5,

TRL/IRL 7 and TRL/IRL 13 [21]. It was later found that other viral and cellular transcripts are also non-specifically incorporated into virions as well as non-infectious enveloped particles and dense bodies [8,22]. Packaging of transcripts into virions has been observed in different HCMV strains [23] and in Herpes Simplex-1 virus [24].

Initially, the detection of HCMV late transcripts UL21.5 and UL65 (pp67) or immediate early transcript UL123 (p72) in leukocytes or whole blood was proposed for monitoring HCMV infection [5,6,25]. HCMV transcripts can be detected in leukocytes or plasma. The HCMV late transcripts found in leukocytes are uptaken from infected endothelial cells, while the immediate early transcripts are both uptaken and synthesized in the leukocytes [26]. Transcripts found in plasma may be released after the lysis of infected cells or can be packaged in virions. Recently, Piccirilli and colleagues reported the presence of UL21.5 mRNA in plasma of transplant recipients with active HCMV infection [27]. Our aim was to extend this observation and define the form in which UL21.5 mRNA is present in plasma, either as RNA packaged in virions or as free-floating RNA. Using the RNAse protection assay, we observed that most of the UL21.5 mRNA in plasma is resistant to RNAse digestion, proving that it is mainly packaged in virions. Indeed, the median reduction in the amount of UL21.5 mRNA after RNAse treatment was within 0.5 Log10 (i.e. less than 30%), with a median reduction of 0.1 Log10. Therefore, although mRNA can also be released in plasma by disrupted cells, it is likely that naked mRNA potentially released from infected cells is degraded by endogenous plasma ribonuclease activity [28]. If it were not the case, the further *ex vivo* treatment of plasma with RNAse would have dramatically reduced the amount of UL21.5 mRNA with respect to that detected before RNAse treatment. Thus, UL21.5 mRNA in plasma is mostly virion-associated and represents a marker for productive HCMV infection leading to the release of virions in plasma. A discrepancy between HCMV DNAmia and UL21.5 mRNA in plasma was observed in the five patients here examined with DNAemia blips associated with an abortive replications during LTV prophylaxis. In these cases, DNAemia breakthrough was not associated with resistance to antiviral drugs and resolved spontaneously. Determination of UL21.5 mRNA gave negative results in these patients. Conversely, it was always positive in case of HCMV infection non-responsive to LTV or MBV because of drug resistance. This further indicates that the presence of UL21.5 mRNA in plasma is associated with productive infection.

Although UL21.5 mRNA in plasma appears virion-associated, we found a weak correlation between mRNA levels and viremia. However, it should be considered that the viremia assay measures the number of leukocytes carrying infectious virus (it is not possible to isolate the virus from plasma), whereas the UL21.5 mRNA in plasma is determined as a marker for virions released in the extracellular compartment. In addition, the viremia assay is poorly standardized and is affected by blood transport and storage conditions.

Plasma UL21.5mRNA levels correlate with WB and PL DNAemia. In addition, it appears in plasma 1-2 weeks prior to preemptive therapy and is usually detectable when WB DNAemia is [?]10,000 copies/ml or PL DNAemia is pre-emptive therapy in hematopoietic stem cell transplant recipients [29]. Therefore, although it appears less sensitive than DNAemia in detecting HCMV in blood, UL21.5 mRNA in plasma may be considered a useful parameter for identifying patients in need of pre-emptive antiviral treatment, since all patients requiring antiviral treatment here analysed were positive for mRNA in plasma some days before the initiation of treatment. We can hypothesize that a potential strategy involving this parameter for initiation of pre-emptive therapy would not lead to a delayed start of treatment.

The limitation of this study is its retrospective nature and relatively small sample size. However, UL21.5 mRNA was detected in all patients with clinically significant DNAemia, and RNAse-resistant mRNA was detected in almost all samples analyzed.

In conclusion, our results suggest that UL21.5 mRNA in plasma is virion-associated and represents a marker for productive HCMV infection. Prospective studies are warranted to verify whether UL21.5 mRNA alone or in combination with HCMV DNAemia can improve current strategies for the treatment of HCMV infection in immunocompromised patients.

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## FIGURE LEGENDS

**FIGURE 1.** A) Levels of HCMV DNA in whole blood (WB DNAemia), HCMV DNA in plasma (PL DNAemia) and PL UL21.5 mRNA in episodes of clinically significant DNAemia (requiring pre-emptive therapy) or transient self-resolving DNAemia. B) Median kinetics of WB DNAemia, PL DNAemia and PL UL21.5 mRNA. Data were normalized by considering 100% the peak value of viral load detected by different assays the day of starting therapy (day 0) and then referring to it as percentages all values detected by the relevant assay either prior to or after onset of therapy. C) Clearance of WB DNAemia, PL DNAemia and PL UL21.5 mRNA after treatment.

**FIGURE 2.** A) Levels of human Rhinovirus (HRV) RNA; B) levels of UL21.5 mRNA in purified preparations of AD169 and VR1814 strains; and C) levels of UL21.5 mRNA in plasma, before or after ribonuclease (RNAse) treatment.

**FIGURE 3.** Pearson's correlation between UL21.5 mRNA in plasma and A) whole blood (WB) DNAemia; B) plasma (PL) DNAemia; C) PL DNAemia after DNAse treatment (i.e. virion-associated PL DNAemia); and C) number of leukocytes carrying infectious virus.

**FIGURE 4.** Levels of A) whole blood (WB) DNAemia or B) plasma (PL) DNAemia in samples positive or negative for UL21.5 mRNA.

**FIGURE 5.** Levels of HCMV DNA in whole blood (WB DNAemia) and plasma (PL) UL21.5 mRNA in episodes of DNAemia associated with resistance to letermovir (LTV) or maribavir (MBV) or in episodes of transient self-resolving DNAemia blips in the absence of resistance to antiviral drugs.

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**TABLE 1.** Characteristics of the patients analysed.

| Characteristics | |
|---|---|
| Sex (M/F) | |
| Median age (IQ range) | |
| Underlying condition (no.) Kidney transplant Lung transplant Heart transplant Hematopoietic stem cell transplant Hemato | |
| Median (IQ range) copies/ml at the beginning of pre-emptive therapy or peak value WB DNAemia x$10^3$ PL DNAemia x$10^3$ | |

WB, whole blood; PL, plasma.

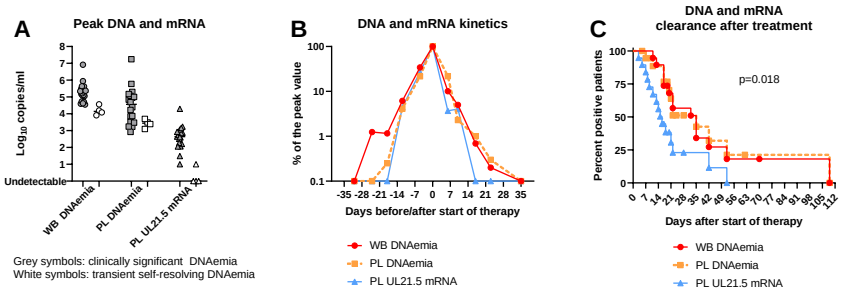



**Figure 1**

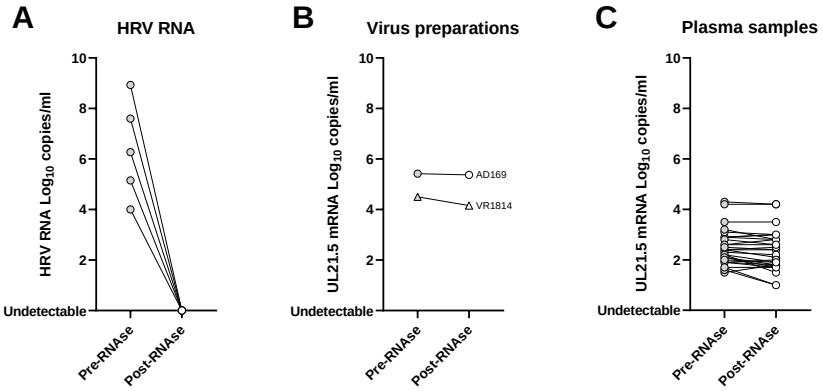



**Figure 2**

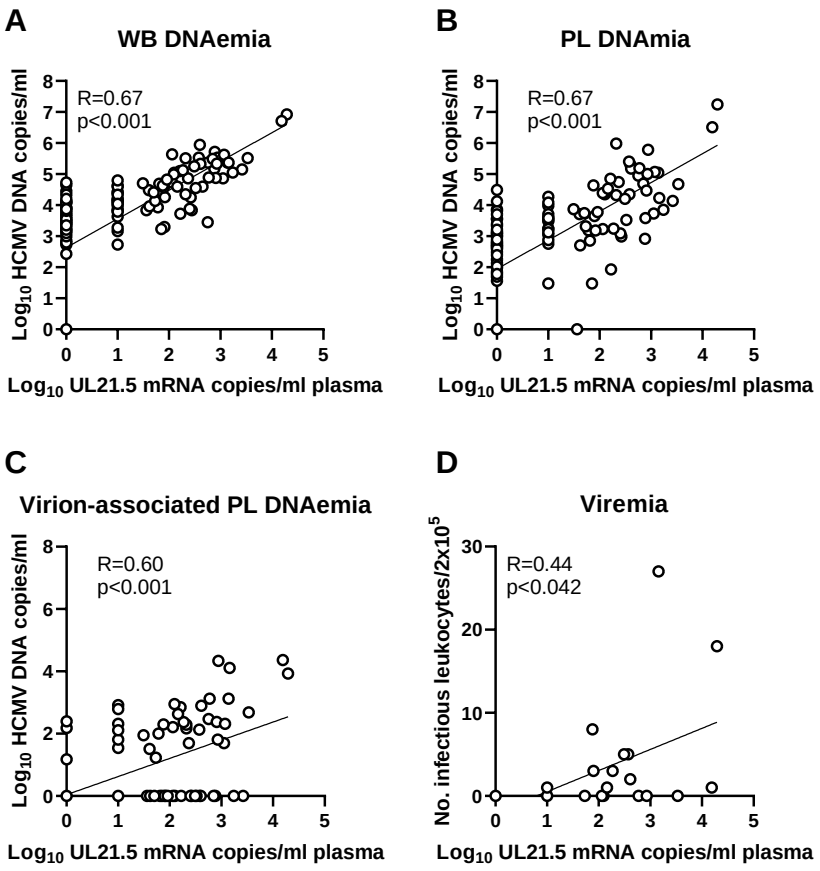



**Figure 3**

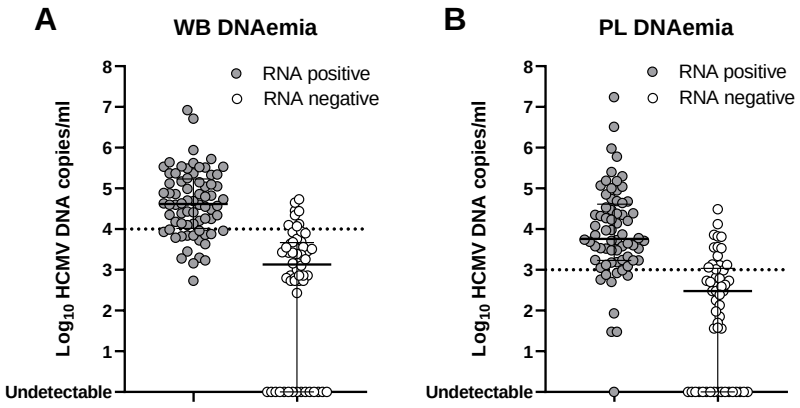
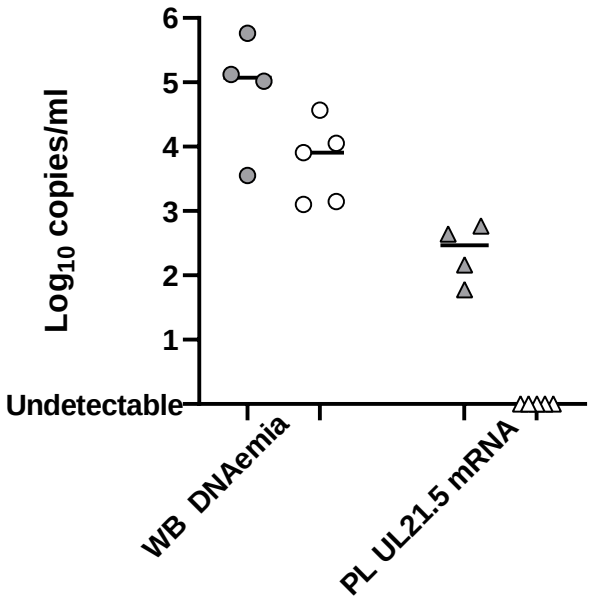



**Figure 4**




Grey symbols: LTV/MBV resistance
White symbols: self-resolving DNAemia during LTV

# Figure 5