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Cytomegalovirus-RNA Accurately Identifies Clinically Significant Infection Needing Preemptive Therapy in Liver Transplanted Children: A Proof-of-Concept Study

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Keywords: Cytomegalovirus | epidemiology | immune system | infection | latent infection | pathogenesis | transplantation | virus classification

ABSTRACT

Preemptive therapy (PET) is safe and effective in controlling Cytomegalovirus (CMV) infection after pediatric liver transplantation (LT) and allows to observe the kinetics of quantitative CMV-DNA viral load till it reaches the treatment thresholds. While early detection of low-to-moderate CMV-DNA levels may not indicate active viral replication, awaiting the viral load to exceed the treatment threshold may lead to viremic breakthroughs and CMV disease. We assessed the capacity of quantitative CMV-RNA (UL21.5 mRNA) to identify active viral replication and its accuracy in identifying clinically significant CMV infection (csCMVi) needing PET in LT children. One-hundred and forty-four comparative quantitative CMV-RNA and CMV-DNA determinations were obtained from 12 children followed prospectically for 6 months after LT. Of 52 CMV-DNA-positive specimens, 17 (32%) were also CMV-RNA-positive, while CMV-RNA was undetectable in CMV-DNA-negative specimens. All children with csCMVi had early detectable CMV-RNA, peaking simultaneously to CMV-DNA (median CMV-DNA: 65 906 cp/mL; median CMV-RNA: 767 cp/mL); conversely, none of those with persistently low DNAemia proved CMV-RNA-positive. In this first pilot study, CMV-RNA had 100% sensitivity and specificity in identifying children needing PET after pediatric LT. The early detection of CMV-RNA marks significant CMV infection/reactivation, thus allowing to avoid unnecessary antiviral treatment.

Abbreviations: CMI, cell-mediated immunity; CMV, Cytomegalovirus; csCMVi, clinically significant CMV infection; D, donor; GCV, ganciclovir; HSCT, hematopoietic stem cell transplantation; LT, liver transplantation; PET, preemptive therapy; R, recipient; VGCV, valganciclovir.

Marco Enrico Giovanni Arosio and Lorenzo D'Antiga contributed equally to this study.

1 | Introduction

Cytomegalovirus (CMV) infection is a major threat in solid organ transplantation and has been associated with a threefold increased risk of graft loss or mortality [1]. Besides its direct cytopathic damage, indirect effects include acute and chronic rejection, thrombotic events, opportunistic infections, and lymphoproliferative disorders triggered by CMV-induced immunoinflammatory changes [2].

Children undergoing liver transplantation (LT) are particularly susceptible to CMV-related complications, being often naïve recipients of CMV reservoir organs. Prophylaxis with Ganciclovir (GCV) or Valganciclovir (VGCV) prevents early CMV infection but bears the risk of side effects – including neutropenia in up to 55% of children – and delayed infections/reactivations considered to increase immunologic graft injury [3, 4]. Preemptive therapy (PET) – consisting of administering antiviral treatment in patients reaching an established viremic threshold – is as effective as prophylaxis in mitigating the CMV burden with lower drug exposure, and is increasingly adopted as an alternative strategy [5, 6].

The main outcome determinants of CMV viremias under PET protocols are (i) the CMV-specific host immune response and (ii) the entity and kinetics of the viremia itself.

CMV-DNA determination by quantitative PCR has by far replaced pp65 antigenemia to monitor viremia in children managed with PET protocols after LT due to its higher sensitivity and reliability also in patients with severe neutropenia, shorter turnaround time, and possibility to process simultaneously a large number of specimens [7]. However, the viral load detected as CMV-DNAemia could represent a free viral genome released from cells or tissues rather than infectious viral bodies, as suggested by the relatively longer persistence of CMV-DNA in plasma (cell-free) compared to whole blood (cell-free and intracellular) specimens of hematopoietic stem cell (HSCT) and kidney transplant recipients [8–10]. Moreover, up to 25% of CMV-DNA-positive LT recipients have no detectable pp65 antigenemia [7]. On the other hand, waiting for the patient to meet an established threshold for treatment may lead to a viremic breakthrough with very high replication and the risk of organ injury.

Comparative monitoring of a CMV-specific mRNA transcript has shown promising accuracy in differentiating between true CMV infection and abortive CMV-DNAemia in solid organ transplantation and HSCT adults treated with Letermovir, a terminase complex inhibitor that leads to the release of inactive viral DNA fragments [11, 12].

No study has investigated the concomitant presence of CMV-DNA and CMV-RNA in pediatric LT recipients nor assessed their respective roles in the infection course and outcomes.

The aim of this study was to assess the capacity of plasma CMV-RNA determined via quantitative reverse transcriptase to identify children with active CMV replication leading to clinically significant CMV infection (csCMVi), namely those needing PET to control the infection.

2 | Methods

This is a prospective observational single-center proof-of-concept sub-study in the framework of the TAILOR-CMV project (T-cell Adaptive Immunity in Liver Organ Recipients infected by CytoMegaloVirus).

Each patient was followed with scheduled visits for the first 6 months after LT (end of study). Informed consent, demographics, physical examination, standard laboratory tests, graft- and non-graft-related complications were recorded on dedicated electronic case report forms.

Patients between 0 and 17 years of age, recipients of LT for any indication not affecting immune function, were enrolled, including early retransplantation (by 1 month). Exclusion criteria were previous antineoplastic chemotherapy, retransplantation between 1 and 12 months since previous LT, and administration of anti-thymocyte globulins.

Virologic monitoring was performed immediately before LT and once a week in the first 12 weeks or in presence of ongoing viremia, then at +4, +5, +6 months, and consisted in (i) quantitative CMV-DNA assay through qPCR on whole blood, and (ii) quantitative CMV-RNA using the ELITe MGB kit on ELITe InGenius instrument (ELITechGroup) targeting the virion-associated UL21.5 mRNA, a late transcript highly expressed during lytic infection [13–15].

CMV-DNA was extracted and quantified from whole blood, with a detection limit of 156 copies (cp)/mL as previously published [16], while CMV-RNA was directly tested in plasma samples with a detection limit of 30 cp/mL. The CMV nucleic acids were expressed as cp/mL, although in clinical use, the CMV-DNA on whole blood is reported as International Units (IU)/mL, using a locally established conversion factor of 0.7 [17].

According to donor (D)/recipient (R) serologic CMV-IgG match, the patients' CMV risk was classified as (i) high (D+/R− or D+/R_{any} in children aging less than 18 months); (ii) medium (D_{any}/R+); (iii) low (D−/R−).

CMV-cell mediated immunity (CMI) was measured through the T-SPOT CMV kit (Oxford Immunotech) against CMV pp65 and IE-1 antigens, measuring Interferon-γ to detect CD8+ and CD4+/CD8+ T-cell response, respectively, before transplantation and at +2, +4 weeks, and +6 months post-LT. Peripheral blood mononuclear cells were freshly isolated through a CPT separation tube and processed according to the manufacturer's instructions. The cut-offs were 30 and 10 spots/200 000 cells for pp65 and IE-1, respectively.

Patients were managed as per previously published local PET protocol [5]. Children received PET in presence of the following conditions: (i) ≥ 100 000 CMV-DNA IU/mL (corresponding to 142 800 cp/mL) on whole blood, or (ii) ≥ 50 000 IU/mL CMV-DNA (71 400 cp/mL) on whole blood in presence of a 10-fold increase in two consecutive measurements in patients less than 1 year age or ≥ 1 year

age and CMV IgG-negative (high-risk patients). PET consisted in GCV 5 mg/kg/dose q12 h for at least 2 weeks and until negative CMV-DNA was confirmed on two consecutive specimens. Patients with stable reduction of CMV-DNA after at least 2 weeks of GCV therapy could be switched to oral therapy with VGCV 15 mg/kg/dose q12 h until negative CMV-DNA was confirmed on two consecutive specimens collected at least 3 days apart.

The pediatric liver transplant team was blinded to the CMV-RNA results all over the study.

CMV outcomes are defined as per consensus nomenclature [18]. CMV disease was defined as detectable viremia in the presence of consistent symptoms, such as CMV syndrome (fever, malaise, and myelosuppression) or proven CMV tissue-invasive disease (by culture, immunohistochemistry, or in situ hybridization with relevant histologic features). CMV disease was an absolute indication of antiviral treatment with the same protocol as per PET.

The composite outcome "clinically significant CMV infection (csCMVi)" was defined by the occurrence of the criteria for PET or CMV disease.

The Student *t*-test, the χ^2 method, or Fisher's exact test were performed when appropriate for statistical analysis to compare continuous and categorical variables. *p* Value less than 0.05 was chosen as cut-off for significance. Data were analyzed with SPSS (IBM Corp. Released 2011). The PSPP Statistics for Mac, Version 1.6.2-2 (2022 Free Software Foundation, GNU General Public License) and GraphPad Prism (GraphPad Prism Version 10.0.3 for Mac, 2023 GraphPad Software, San Diego, CA) softwares were used for statistical analysis.

The TAILOR-CMV study was approved by the Ethics Committee of Bergamo with the protocol n. 190/20 on September 7, 2020.

3 | Results

Twelve children (7 females; median age at LT 3.2 years, range 5 months to 16 years) were enrolled between May 2023 and March 2024. The liver graft type was a left lateral segment in 7 (two of which were from living donors), a right lobe in 4, and a whole organ in 1. The primary liver disease was biliary atresia in 6, cystic fibrosis in 2, autoimmune hepatitis, choledocal malformation, and progressive familial intrahepatic cholestasis type 3 in 1 each; the remaining patient received a re-transplantation for ischemic cholangiopathy of the previous graft. Standard immunosuppression consisted of corticosteroid induction + tacrolimus for all, while mycophenolic acid was added in one patient in the context of a lower dose tacrolimus regimen for renal protection. All patients completed the study, and basal characteristics, as well as main infectious and transplant outcomes, are shown in Table 1. Infections other than CMV occurred in six patients and included central line-associated bloodstream infections in two and urinary tract infections, HSV1, Adenovirus, and primary EBV infection in one patient each.

TABLE 1 | Basal characteristics and main infectious and transplant outcomes of the 12 liver-transplanted children included.

Gender, *N* (%)	
M	5 (42)
F	7 (58)
Age at LT (years), median (range)	3.2 (0.4–16.4)
Liver graft type, *N* (%)	
Left split	7 (58)
Right split	4 (33)
Whole liver	1 (9)
CMV risk, *N* (%)	
Low	0
Medium	7 (58)
High	5 (42)
CMV outcome, *N* (%)	
No infection	3 (25)
Infection, low viremia	5 (42)
csCMVi	4 (33)
Peak CMV-DNA	
IU/mL, median (range)	5097 (266–276 914)
cp/mL, median (range)	7281 (380–395 591)
CMV-DNA cumulative duration (days), median (range)	36.5 (0–186)
CMV-RNA positivity, *N* (%)	
Positive	4 (33)
Negative	8 (67)
Peak CMV-RNA (cp/mL), median (range)	1103.5 (20–6550)
CMV-RNA cumulative duration (days), median (range)	0 (0–96)
TCMR, *N* (%)	
Yes	2 (17)
No	10 (83)
Biliary complications, *N* (%)	
Yes	3 (25)
No	9 (75)
Vascular complications, *N* (%)	
Yes	2 (17)
No	10 (83)
Other infections, *N* (%)	
Yes	6 (50)
No	6 (50)

Abbreviations: CMV, Cytomegalovirus; csCMVi, clinically significant CMV infection; LT, liver transplantation; TCMR, T-cell mediated rejection.

Of the 144 blood specimens obtained at the moment of the LT and until +12 weeks after LT, 52 (36%) were CMV-DNA-positive. Of these CMV-DNA-positive specimens, 17 (32%) resulted to be also CMV-RNA-positive, while CMV-RNA was not detectable in any of

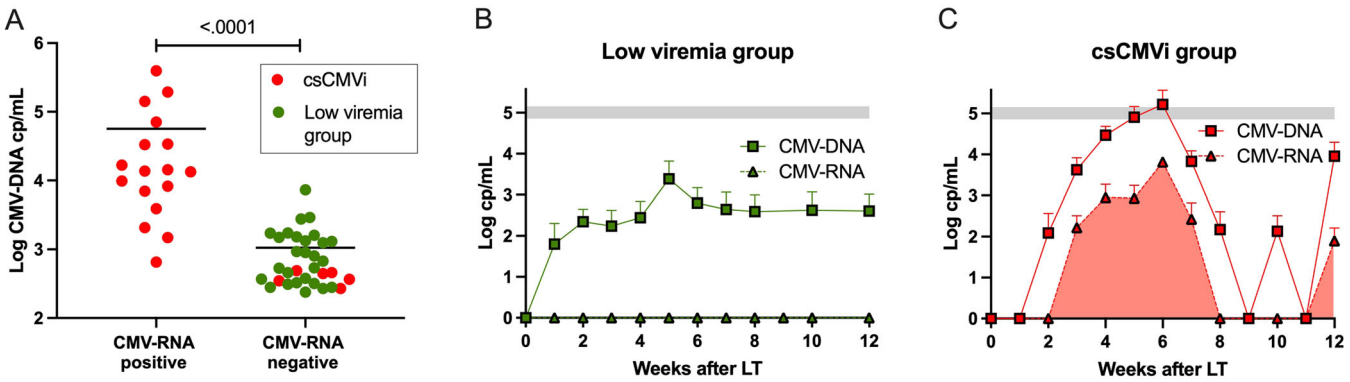

FIGURE 1 | (A) Scatter plot of the Log CMV-DNA according to the concomitant CMV-RNA result. Red dots are specimens from patients in the "clinically significant CMV infection" group, and green dots from patients in the "Low viremia" group. (B) and (C) Kinetics of the Log CMV-DNA (squares, solid line) and CMV-RNA (triangles, dotted line) expressed as mean ± standard deviation in "Low viremia" and "clinically significant CMV infection" groups. The gray area represents the threshold adopted for preemptive therapy. csCMVi, clinically significant CMV infection; LT, liver transplantation.

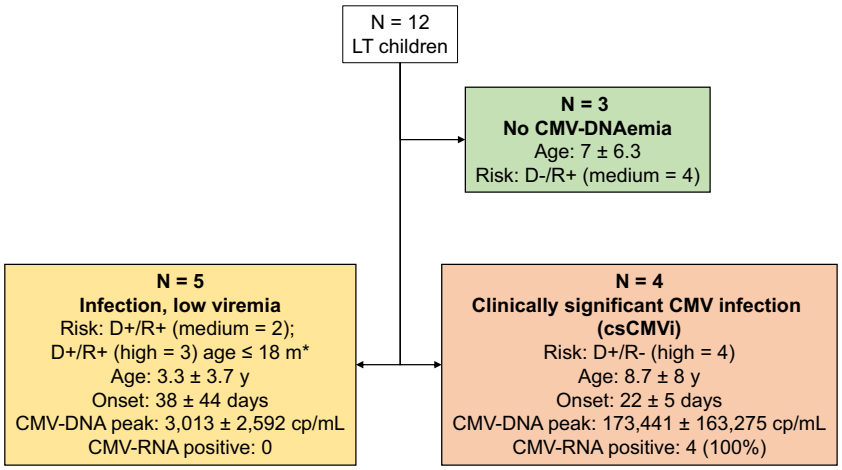

FIGURE 2 | Flow chart displaying the CMV-related outcome of the 12 patients included. *Children aging ≤ 18 months at liver transplant, in whom CMV-IgG positivity due to maternal antibodies cannot be excluded. D, donor CMV-IgG; LT, liver transplantation; R, recipient IgG.

the CMV-DNA-negative specimens. CMV-DNA was significantly higher in CMV-RNA-positive (56 499 ± 102 577 copies/mL) than in CMV-RNA-negative specimens (1189 ± 1382; $p < 0.0001$) as shown in Figure 1A. Among the group with csCMVi, 6 (26%) of the CMV-DNA-positive specimens proved CMV-RNA negative, being those collected in the very early, low DNAemic phase, and belonging to patients in whom CMV-RNA would subsequently become detectable before the antiviral therapy.

The patients' outcome according to CMV is shown in Figure 2. Three out of 12 children did not present any positive CMV-DNA after LT. Of the remaining 9 children, 5 (55%) maintained a low viremia below the threshold for the PET, with no symptoms of CMV disease; the remaining 4 (45%) had a csCMVi, consisting of high-viremic infection reaching the threshold for PET in 3 children, and CMV colitis in one. The four patients with csMCVi were promptly and successfully treated with GCV iv for a median time of 29 (19–47) days. Of note, pulse steroids for T-cell mediated rejection were needed in two patients overall, and in one of them preceded by 12 days the occurrence of breakthrough CMV-DNAemia leading to PET.

No association was observed between the CMV infection fate and age, graft type, or graft-related complications. As expected, high-risk (D+/R−) serostatus match was associated with the CMV infection outcome, being more frequent in csCMVi (100%) than in low-viremic or non-viremic children (60% and 0%, respectively; $p = 0.029$).

Interestingly, CMV-RNA had the strongest association with the CMV outcome and 100% sensitivity (95% CI: 39.7%–100%) and specificity (95% CI: 47.8%–100%) in identifying the need for PET since it was detectable in all children with csCMVi whereas it was undetectable in non-viremic and low-viremic children (Figure 1B,C). Also importantly, in the ascending phase CMV-RNAemia occurred with little or no lag time to CMV-DNAemia (median 0 days, range 0–6 days) and peaked simultaneously with a median peak CMV-DNA equal to 65 906 cp/mL (IQR: 12 094–321 979), and a median peak CMV-RNA of 767 cp/mL (IQR: 59–1749) at around 6 weeks after LT. After treatment initiation, in the descending phase, negative CMV-RNA results tended to occur earlier (median: −6 days) than CMV-DNA clearance.

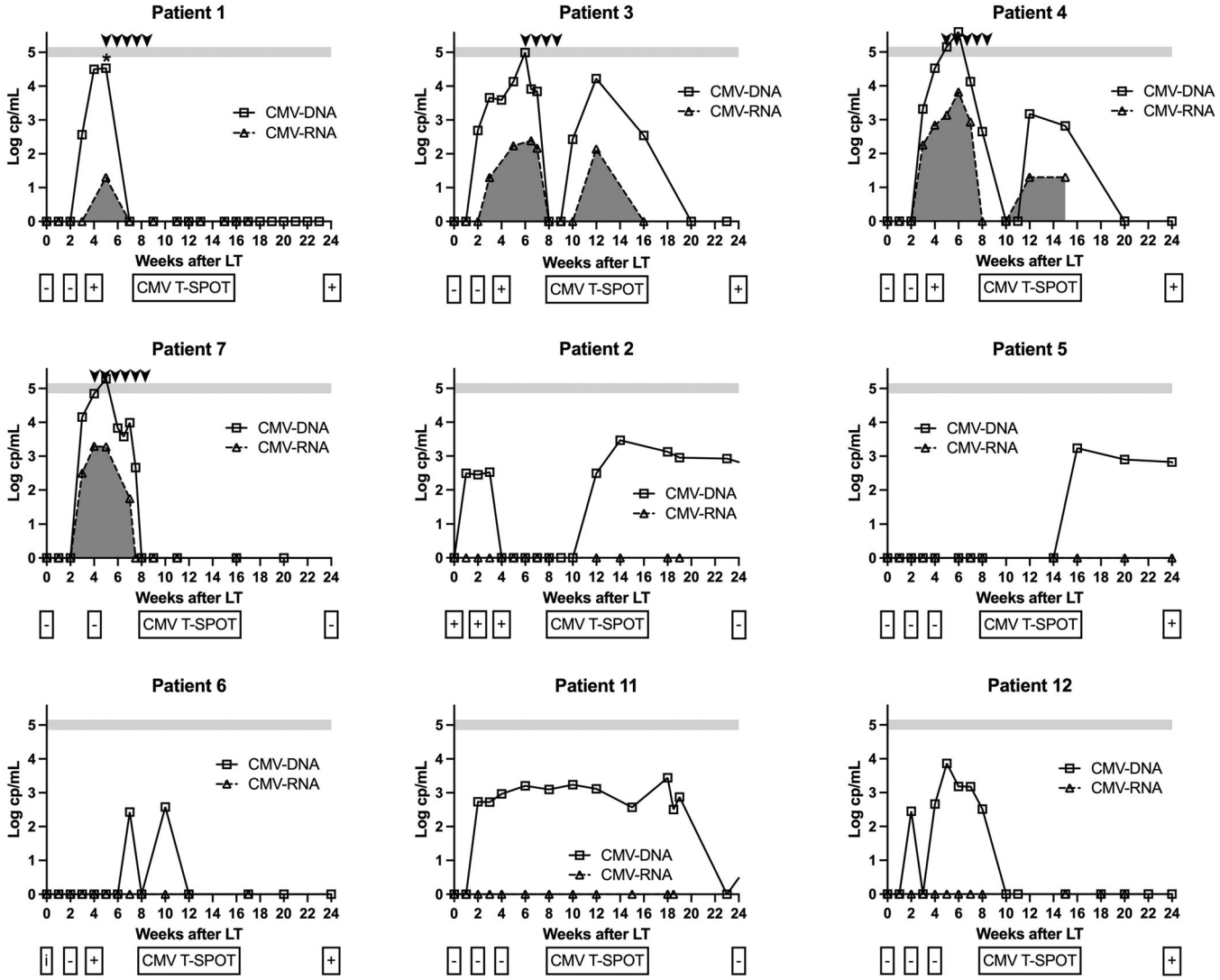

FIGURE 3 | Monitoring of CMV viremia in 9 CMV-DNA-positive children after liver transplantation. Patients 1, 3, 4, and 7 display clinically significant CMV infection (Patient 1 = CMV disease; Patients 3, 4, and 7 = high-viremic infection treated with preemptive therapy); patients 2, 5, 6, 11, and 12 maintained low viremia and did not require preemptive therapy. Log CMV-DNA (squares, solid line) and CMV-RNA (triangles, dotted line) are shown; arrowheads represent the GCV treatment; CMV T-SPOT results at 0, +2, +4 weeks, and +6 months are considered as positive (+) or negative (−) with regard to the threshold of ≥ 30 or ≥ 10 spots/200 000 cells for pp65 or IE-1, respectively, while specimen failing the positive control are indetermined (i); the gray area represents the threshold adopted for preemptive therapy; *diagnosis of CMV disease (CMV-related colitis). LT, liver transplantation.

Figure 3 shows the kinetics of CMV nucleic acids during the viremia episodes in actively infected children with csCMVi (Patients 1, 3, 4, 7) and in noncomplicated low-viremic children (Patients 2, 5, 6, 11, 12). CMV-RNA was detectable in the presence of initial ascending CMV-DNAemia as low as 366, 4531, 2077, and 14 307 cp/mL in children who would later meet the criteria for PET. Among the children with csCMVi, two remained steadily CMV-DNA and CMV-RNA free after antiviral treatment (Patients 1 and 7). Conversely, Patients 3 and 4 had a replication relapse marked by a second CMV-DNAemia and CMV-RNAemia, which spontaneously resolved, and both patients exhibited acquisition of CMV-specific CMI as judged by the positive T-SPOT at +4 weeks and +6 months. Overall, the acquisition of CMV-CMI was documented in 3 (75%) of the csMCVi group and in 2 (40%) of the low viremia group.

4 | Discussion

This is the first prospective study exploring the usefulness of CMV-RNA monitoring in addition to CMV-DNA in children managed with PET after LT. A permissive protocol with a high threshold for treatment represents an ideal framework to test the capacity of a viral mRNA transcriptional assay in documenting active replication and thus predicting high-viremic complicated CMV infection.

Indeed, we observed that the presence of detectable CMV-RNA in CMV-DNA-positive children heralds a logarithmic increase in the viral load, thus clearly identifying children with csCMVi, needing antivirals as PET or to treat tissue-invasive CMV disease, with 100% sensitivity and specificity. All the patients with csCMVi (high/logarithmic viremia, CMV disease) had an early

positivization of CMV-RNA along with CMV-DNA, while none of those spontaneously clearing the CMV-DNAemia had a concomitant RNA positivity.

CMV-specific mRNA transcripts – including that of the UL21.5 gene – mark the ongoing lifecycle of the pathogen, promoting secondary gene expression, viral DNA replication, and ultimately, virion assembly [19]. Thus, CMV-RNA positivity indicates active viral replication and true, potentially life- and graft-threatening infection that needs to be promptly tackled. Conversely, CMV-RNA-negative DNAemia might be due to a passive release of viral genome fragment degradation from cells and tissues – especially the major CMV reservoirs like liver graft, bone marrow or gut – following infectious, immunoinflammatory, or ischemic injury, the so-called "abortive" infection.

This concept is also strengthened by (i) the fact that CMV-RNAemia occurs within the CMV-DNAemia episodes; (ii) the fact that CMV-RNA kinetics parallels that of CMV-DNA in each episode/patient; (iii) the capability of the CMV-RNAemia episodes to trigger CMV-specific CMI as judged by T-SPOT.

In a prospective study on 21 adult heart and lung transplant recipients – mostly with non-high risk – managed with a hybrid prophylaxis + PET protocol using as a gold standard for treatment pp65 antigenemia (with a threshold of ≥ 2 or ≥ 5 pp65-Ag-positive leukocytes/50 000 leukocytes), quantitative pp67-mRNAemia did not prove accurate enough for decision making, with sensitivity and specificity of 61% and 83%, respectively [20]. Conversely, in another study on adult thoracic organ recipients, pp67-mRNA had higher sensitivity than pp65-antigenemia in driving PET, and this difference might be explained by the at least fivefold higher threshold (100 pp65-Ag-positive leukocytes/200 000 polymorphonuclear cells) used for PET [21].

A retrospective report focused on the performance of the quantitative UL21.5-mRNA (the same used in the present study) in 47 CMV-DNA-positive specimens from 44 solid organ recipients [11]. The authors claimed that CMV-RNA allowed recognition of residual replication in 14% of patients under Letermovir prophylaxis, while CMV-RNA positivity marked active replication in 26% and 57% of the patients under Letermovir off-label treatments as per GCV-resistant strains, and of the unselected group undergoing PET, respectively. The authors concluded that CMV-RNA could be of clinical usefulness in monitoring patients under Letermovir prophylaxis, but they did not test its performance in the real-life PET scenario.

Our pilot experience demonstrates a high accuracy of the CMV-UL21.5-mRNA in early identifying csCMVi, suggesting that CMV-RNA could be a promising instrument to monitor and interpret CMV viremia, and that initiating antivirals in LT children on its positivity could lead to a timely intervention with no risk of overtreatment. If the criterion for initiating PET had been CMV-RNA positivity, treatment initiation would have been anticipated by a median of 2 (range 1–3) weeks. If these results are confirmed on larger cohorts, CMV-RNA-driven PET protocols could allow earlier treatments, faster viral clearance, less recurrence, and less risk of drug resistance, meaning safer management.

On the other hand, there is too limited data to consider CMV-RNA negativization as a criterion for stopping the antiviral treatment. Evidence from solid organ transplanted adults treated for CMV disease clearly indicates that residual CMV-DNA positivity implicates a substantial risk of clinical and virologic recurrence [22]. Confirmed negativization of the CMV-DNA should remain the standard criterion for stopping the treatment after its initiation for therapeutic and preemptive purposes.

The limitations of these observations are the relatively little number of patients and the relatively limited time of CMV-RNA monitoring. Moreover, although these cases are rather representative of the pediatric LT population, the two groups ("csCMVi" and "low viremia group") were also neatly different according to the basal serologic and immune risk and exhibited markedly different CMV-DNA kinetics. Larger studies will allow us to confirm the usefulness of the CMV-RNA in children with low serologic risk (pre-LT positive CMV-IgG) but persistent moderate-to-high DNAemia. Further studies will also define the role of CMV-RNA monitoring in assessing the response to treatment in complex cases, especially when genetic antiviral resistance is suspected. Nonetheless, this preliminary experience suggests that CMV-RNA may become the main marker of CMV active infection in immunosuppressed patients, and guide the decisions to treat only those at risk of CMV disease and its complications.

Author Contributions

Study conception and design, interpretation of the data, drafting the work: Emanuele Nicastro, Eleonora Severi, Lorenza Matarazzo, Marco Enrico Giovanni Arosio, and Lorenzo D'Antiga. Analysis and interpretation of the data and reviewing the draft: Francesco Morotti, Alessandra Tebaldi, Ezio Bonanomi, Michela Bravi, Angelo Di Giorgio, Samuele Covini, Marta Dolci, and Domenico Pinelli. Acquisition of the data and reviewing the draft: Ilaria Passera, Michele Totaro, and Laura Fornataro.

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Data Availability Statement

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

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