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Cytomegalovirus infection and drug resistance emergence during letermovir salvage therapy in a pediatric SCID patient

Fien Horsten[1], Sarah Gillemot[1], Pierluigi Calò[2], Pauline Mazilier[2], Piet Maes[3], Robert Snoeck[1] & Graciela Andrei[1] ✉

Cytomegalovirus (CMV) infection is a common complication in newborns with severe combined immunodeficiency (SCID). Prolonged antiviral treatment in immunocompromised patients increases the risk of the emergence of drug resistance. We analyzed drug resistance in a newborn with SCID who developed neonatal CMV infection. Sequencing of viral DNA polymerase (DP; *UL54*), protein kinase (*UL97*), and terminase (*UL51*, *UL56*, *UL89*) genes identified ganciclovir (GCV) and foscarnet (PFA) resistance mutations in blood, but not cerebrospinal fluid. After treatment was shifted to cidofovir and letermovir (LMV), a LMV resistance mutation rapidly emerged in *UL56* (C325F). Eventually, a multidrug-resistant genotype was established (DP-V781I and UL56-C325F). Whole-genome sequencing of CMV in clinical blood samples showed an otherwise stable genotype. This case describes a CMV infection complicated by compartmentalization and the emergence of resistance to GCV, PFA, and LMV. It highlights the need for further investigation into alternative antiviral strategies for the prevention and treatment of CMV.

Cytomegalovirus (CMV) infection can cause severe morbidity or mortality in infants with severe combined immunodeficiency (SCID)[1,2]. After maternal immunity wanes, infants with SCID are left vulnerable due to their impaired adaptive immunity, putting them at risk of severe disseminated CMV infection presenting, for example, as pneumonia, hepatitis, retinitis, neutropenia, thrombocytopenia, or central nervous system infection[1].

SCID is a group of rare genetic disorders leading to a primary immunodeficiency (PID) that is characterized by the absence of functional T lymphocytes (T-), with possible disruption of B lymphocyte and/or natural killer (NK) cell function[3]. Currently, over 20 genetic defects are known to cause SCID and the incidence is estimated between 1:40,000 and 1:100,000 live births[4–7]. If left untreated, SCID is typically fatal in the first two years of life as a result of opportunistic infections stemming from the lack of a functional immune system. T-cell disorders are often characterized by pneumonia with *Pneumocystis jirovecii* or CMV, mycobacterial infection, recurrent skin candidiasis, or diarrhea.

The main curative treatment option for SCID is hematopoietic stem cell transplantation (HSCT)[8]. Hygiene measures are essential to prevent infection before HSCT, as active infections at the time of transplantation lower the chances of successful immune reconstitution and patient survival[8,9]. In newborns with SCID, CMV transmission through breast milk from CMV-seropositive mothers is a concern which can be addressed by advising mothers to withhold breastfeeding, although further research is needed to clearly define the risks[10–12].

Antivirals against CMV include ganciclovir (GCV), its prodrug valganciclovir (VGCV), cidofovir (CDV), and foscarnet (PFA), all targeting the viral DNA polymerase (DP). Nucleoside or nucleotide analogs (GCV and CDV, respectively) inhibit DP through competitive inhibition resulting in chain termination during DNA replication, while PFA is a pyrophosphate analog that directly inhibits the DP through binding of the pyrophosphate binding site, disrupting chain elongation. Maribavir (MBV) competitively inhibits the viral protein kinase (PK), preventing its phosphorylating functions and thereby preventing DNA replication, encapsidation, and nuclear egress. Letermovir (LMV) disturbs viral DNA processing and packaging through binding of the UL56 subunit of the viral terminase complex. Prolonged antiviral treatment is often required for immunocompromised individuals, but this approach may facilitate the emergence of drug resistance when viral replication is incompletely suppressed. A 1998 study observed an earlier emergence of drug resistance in CMV infections in pediatric patients with primary combined immunodeficiencies

[1]Molecular, Structural and Translational Virology Research Group, Rega Institute, Department of Microbiology, Immunology and Transplantation, KU Leuven, Leuven, Belgium. [2]Department of Pediatric oncology and Bone marrow transplantation, Université Libre de Bruxelles (ULB), Hôpital Universitaire de Bruxelles (HUB), Hôpital universitaire des Enfants Reine Fabiola, Brussels, Belgium. [3]Laboratory of Clinical and Epidemiological Virology, Rega Institute, Department of Microbiology, Immunology and Transplantation, KU Leuven, Leuven, Belgium. ✉e-mail: graciela.andrei@kuleuven.be

than in other CMV-infected populations[13]. The authors stressed the need for resistance surveillance and prompt therapeutic response. A strategy for the prevention of antiviral drug resistance, well-established in the human immunodeficiency virus (HIV) field, is combination therapy. The combined administration of multiple antivirals with different mechanisms of action and/or different viral targets increases the barrier to resistance.

Traditionally, resistance screening consists of Sanger sequencing of genes where resistance mutations are known to appear: DP gene *UL54* (GCV, CDV, PFA resistance), protein kinase (PK) gene *UL97* (GCV, MBV resistance), *UL27* (MBV resistance, in vitro), and terminase subunit genes *UL56*, *UL89*, *UL51* (LMV resistance)[14]. Recent advances in next-generation sequencing (NGS) techniques have driven the consideration of NGS in resistance screening[15]. The increased sensitivity of NGS over Sanger sequencing allows for the detection of minor genetic variants, offering significant clinical benefits. A validated, standardized approach is required for the systematic implementation of NGS in resistance screening.

In this study, we performed a longitudinal analysis of a CMV infection in a pediatric SCID patient treated at the Children's Hospital (HUDERF) of the Brussels University Hospital and followed under our translational research platform RegaVir (www.regavir.org). Longitudinal sampling enabled the analysis of viral responses to treatment and the emergence of resistance. We prospectively studied antiviral resistance through Sanger sequencing to guide clinical decisions. Retrospective targeted NGS and whole-genome sequencing enabled the further analysis of viral adaptation in response to antiviral treatment.

Results

Case presentation and viral genotyping

The patient was born to a CMV-seropositive mother at 39 weeks gestation. The patient's parents were 2nd degree consanguineous, and her family history included several unexplained miscarriages and two siblings who died at a young age (4.5 and 9 months) from infectious complications. The patient was admitted to the hospital 56 days after birth for thrush with ulcerated oral lesions and pyrexia. Immuno-hematological examinations were strongly suggestive of a constitutional immunodeficiency. T-B-NK+ SCID diagnosis was confirmed when the patient was found to be homozygous for a mutation in the gene encoding the RAG2 protein. *P. jirovecii* prophylaxis with trimethoprim-sulfamethoxazole was started.

CMV infection was first detected in urine, likely transmitted through breast milk as CMV PCR from the Guthrie test was negative. The child was weaned from breast milk and GCV treatment was administered from day 71 until day 83 after birth (5 mg/kg 2×/day; Fig. 1). The doses and timing of antiviral drug administration are described in detail in Supplementary Table 1. No intrafamilial human leukocyte antigen (HLA) matched donor was available for HSCT and the severity of the condition did not allow for the delay related to identifying an HLA-matched donor in the registry. Instead, a haploidentical peripheral blood stem cell transplantation (PBSCT) without prior conditioning was performed on day 83 after birth, with the patient's mother as donor. The donor was stimulated with granulocyte colony-stimulating factor (G-CSF) three days prior to PBSCT, and the graft was T-cell depleted by selection of $CD34^+$ cells.

With a CMV viral load of 6 log copies/mL, antiviral treatment was shifted from GCV to PFA (dose varied from 87 mg/kg 2×/day to 97 mg/kg 2×/day) on day 84 to prevent graft failure due to GCV-related hematological toxicity during the engraftment period. Blood CMV viral load had decreased to 5 log at 94 days after birth and the patient showed no clinical symptoms of infection. CMV was first detected in the cerebrospinal fluid (CSF) on day 111 after birth (3 log), indicating CMV meningitis. Three blood samples (B-94, B-108, B-111) and two CSF samples (CSF-111, CSF-117) were prospectively analyzed for genotypical antiviral resistance, showing no known resistance mutations in the *UL54* (DP) and *UL97* (PK) genes (Table 1).

Refractory CMV infection warranted combination therapy of GCV (4.5 mg/kg 2×/day and later 5.1 mg/kg 2×/day) and PFA (82 mg/kg 2×/day and later 89 mg/kg 2×/day) from day 117 after birth. Two doses of G-CSF were administered for GCV-related neutropenia and GCV was discontinued after one month due to leuko-neutropenia. PFA treatment was continued at a dose of 90 mg/kg 2×/day. The patient received a donor lymphocyte infusion (DLI) and later received CMV-specific cytotoxic T

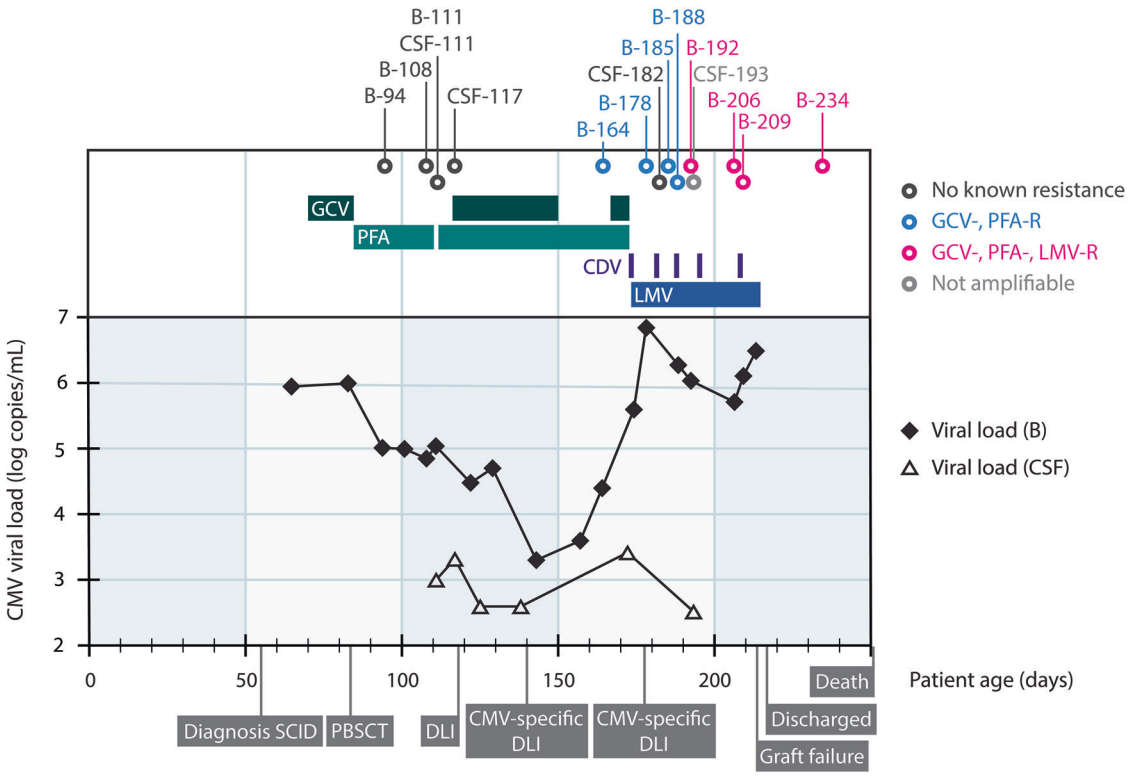

Fig. 1 | Overview of clinical events, antiviral treatment, and CMV viral load (log copies/mL) in a newborn patient with SCID, in relation to the patient's age (in days). Samples were taken from blood (B) and cerebrospinal fluid (CSF). R resistance, PBSCT peripheral blood stem cell transplantation, DLI donor lymphocyte infusion.

Table 1 | Genetic variants detected by prospective Sanger sequencing and variant frequencies detected by retrospective NGS (in brackets ±SD)

Viral isolate	CMV DNA (log copies/mL)	Amino acid changes known to be related to drug resistance					Resistance to:
		PK	DP	pUL51	pUL56	pUL89	
B-94	5.01	none	none	ND	ND	ND	/
B-108	4.85	none	none	ND	ND	ND	/
B-111	5.04	none	none	ND	ND	ND	/
CSF-111	3.01	none	none	ND	ND	ND	/
CSF-117	ND	none	none	ND	ND	ND	/
B-164	4.4	none	V715M[a] (29.56 ± 5.76%)[b] A809V[a] (36.01 ± 10.11%)	ND	none	ND	PFA, GCV
B-178	ND	none	V715M[a] (11.22 ± 1.16%) V781I[a] (13.26 ± 1.58%) A809V[a] (56.80 ± 3.63%)	none	none	none	PFA, GCV
CSF-182	ND	none	none	none	none	none	/
B-185	ND	none	V715M[a] (14.22 ± 2.83%) V781I[c] (8.29 ± 11.72%) A809V[a] (72.34 ± 16.41%)	none	none	none	PFA, GCV
B-188	6.27	none	V715M[a] (63.09 ± 4.24%) V781I[c] (5.56 ± 3.82%) A809V[a] (25.34 ± 8.14%)	none	none	none	PFA, GCV
B-192	6.03	none	V715M[a] (7.24 ± 10.23%) V781I[a] (5.17 ± 7.32%) A809V[a] (74.01 ± 10.51%)	none	C325F[c] (6.48 ± 1.16%)	none	PFA, GCV, LMV
CSF-193	ND	NA	NA	NA	ND	ND	ND
B-206	5.71	none	V781I[a] (91.27 ± 4.36%)	none	C325F (94.50 ± 2.87%)	none	PFA, GCV, LMV
B-209	ND	none	V781I[a] (98.49 ± 0.32%)	none	C325F (99.10 ± 0.10%)	none	PFA, GCV, LMV
B-234	ND	none	V781I (97.21 ± 0.03%)	none	C325F (96.91 ± 0.05%)	none	PFA, GCV, LMV

The genes *UL54* (DNA polymerase, DP), *UL97* (protein kinase, PK), *UL51*, *UL56*, and *UL89* (subunits of viral terminase) were prospectively analyzed by Sanger sequencing while the *UL54* and *UL56* genes were analyzed by Illumina sequencing retrospectively. *UL54* was partially analyzed (amino acid 255 to 1098) while *UL56* was fully sequenced. B, blood; CSF, cerebrospinal fluid; NA, not amplifiable; ND, not done; [a] Heterogeneous population detected by Sanger sequencing; [b] Variant frequency detected by NGS; [c] Variant only detected by NGS.

lymphocytes twice. Nevertheless, CMV viral load in blood gradually increased to 6.88 log on day 178 after birth, with febrile recurrences. CSF viral load remained relatively stable. GCV treatment was reinstated on day 167 at a reduced dose (2.8 mg/kg 2×/day), while awaiting resistance genotyping.

Sample B-164 carried two known resistance mutations in the DP gene: V715M (29.56%; PFA resistance) and A809V (36.01%; GCV and PFA resistance) (Table 1). GCV and PFA were discontinued and replaced with a combination therapy of CDV (5 mg/kg 1×/week; day 173) and LMV (70 mg; day 174). The following blood samples (B-178, B-185, B-188, B-192) showed the persistence of the previously identified mutations DP-V715M, DP-A809V, and the emergence of DP-V781I, known to confer GCV and PFA resistance, as mixed populations. A CSF sample (CSF-182) taken in this period showed no emergence of known resistance mutations, indicative of compartmentalized CMV infection.

Genotyping of samples B-206 and B-209 showed the emergence of the pUL56-C325F (94.50% and 99.10%, respectively), known to confer resistance to LMV. The DP gene now only carried the V781I mutation (91.27% and 98.49%, respectively), as the virus established a stable genotype. Retrospectively, pUL56-C325F was detected in the previous sample B-192 at a frequency of 6.48% due to the increased sensitivity of targeted NGS. At the time, this knowledge would have allowed an earlier evaluation of clinical decisions. Blood viral load increased again to 6.49 log on day 213 after birth, when the parents were informed of graft failure and antiviral escape and palliative supportive care was started before the patient was discharged. Sample B-234 showed the persistent presence of DP-V781I (97.21%) and UL56-C325F (96.91%). The patient died 251 days after birth.

Polymorphisms were shared between CMV detected in blood and in CSF: *UL54* carried S655L, F669L, N685S, L897S, and N898D and *UL56* carried V425A, M442T, T452I, S454N, A476V, N586D, and V778A.

Whole-genome sequencing

Whole-genome sequencing using the target enrichment technique based on RNA probes was performed for all blood samples except B-185, B-188, B-206 (insufficient quantity). The remaining eight samples were sequenced with successful target enrichment and high sequencing coverage (Table 2). Throughout the infection, consensus sequences showed no longitudinal variation over the entire genome except for the emergence of the previously described drug resistance mutations. Manual evaluation of minor variants (5–50% frequency) showed no longitudinal variation in known hypervariable genes [*RL5A*, *UL1*, *UL9*, *UL11*, *UL120*, *UL123*, *UL139*, *UL146*, *UL148D*] and genes encoding glycoproteins [*UL55* (glycoprotein B; gB), *UL73* (gN), *UL74* (gO), *UL75* (gH), *UL100* (gM), *UL115* (gL)][16,17].

Discussion

The longitudinal follow-up of a severely immunocompromised newborn with SCID who developed neonatal CMV infection in the present study enabled the investigation of infection dynamics and development of drug resistance throughout the course of infection. This case highlights the challenge of managing CMV infections and the complexity of antiviral treatment in the pediatric SCID population. Pediatric patients with the primary immune deficiency SCID are extremely susceptible to infection. In this case, the newborn contracted CMV through breastfeeding before HSCT. The active CMV infection at the time of HSCT possibly affected the success of immune reconstitution[8,9]. Antiviral treatment was repeatedly adapted due to toxicity, resulting in an increasingly complex treatment regimen (Fig. 1).

Combination therapies of GCV + PFA and LMV + CDV were employed to address refractory/resistant CMV infection. The clinical application of combination therapy in the treatment of CMV has been described sporadically[18,19]. The 10th European Conference on Infections in Leukaemia (ECIL) supported the consideration of combination therapy for

Table 2 | Sequencing metrics show the effectiveness of target enrichment through quantifying the proportion of reads that map to the CMV reference Merlin or the host (human) genome

Viral sample	Average read length	Reads after trim (no.)	CMV reads (no.)	CMV reads (%)	Human reads (%)	Average coverage	% of reference covered
B-94	154.71	2,559,643	2,004,619	78.32	17.22	1273.06	95
B-108	154.89	2,783,142	2,098,277	75.39	19.11	1314.75	95
B-111	153.28	2,875,892	2,239,323	77.87	17.09	1414.18	96
B-164	151.45	3,635,523	3,125,024	85.96	9.32	1941.64	96
B-178	157.15	5,972,182	5,665,079	94.86	0.28	3633.32	96
B-192	156.14	3,591,942	3,388,119	94.33	1.41	2164.14	96
B-209	151.90	4,359,558	4,124,537	94.61	0.64	2564.83	95
B-234	155.82	7,165,181	6,836,668	95.42	0.32	4366.77	96

second or third line pre-emptive therapy in the pediatric population, as in the ECIL 7 guidelines[20]. A publication describing the ECIL 10 recommendations is anticipated. Combination therapy is most effective when different viral proteins are targeted. While the range of treatment options has expanded with the recent approval of novel anti-CMV drugs targeting viral proteins other than the DP, their safety and efficacy in the pediatric population require further investigation[21–23]. In HSV-1, the combination of GCV and trifluridine, and of GCV or PFA with pritelivir, inhibitors of the viral helicase-primase complex, prevented the emergence of drug resistance mutations in vitro[24,25]. Further research is required to validate this principle in the context of CMV.

In this case, resistance mutations emerged in the genes encoding the viral DP and pUL56. DP-V715M confers PFA resistance and DP-A809V confers PFA- and low-level GCV resistance, but both mutations cause viral growth attenuation[26–28]. In contrast, DP-V781I confers PFA and GCV resistance with no effect on viral fitness[29]. The lack of fitness cost might explain the preferential selection of DP-V781I in this infection. Based on the similar pattern in variant frequencies, the pUL56-C325F substitution appeared to have been selected in the virus carrying the DP-V781I, after which positive selection allowed these mutations to reach fixation in the population as the major viral variant (Table 1).

LMV is currently approved by the European Medicines Agency (EMA) for CMV prophylaxis in adult CMV-seropositive recipients of allogeneic HSCT. Its use as salvage therapy in either adults or the pediatric population is therefore off-label[30,31]. Resistance screening using Sanger sequencing identified the LMV resistance mutation pUL56-C325F in a sample taken 32 days after treatment onset in this patient. Retrospective NGS could already detect this mutation in a sample taken 18 days after treatment onset at 6.48% frequency, illustrating the benefit of the increased sensitivity of NGS. The implementation of NGS in routine resistance screening would require validated thresholds for variant prevalence and a more comprehensive understanding of the clinical relevance of low-frequency variants[32–34]. Until standardized NGS protocols with clearly defined thresholds for clinically relevant low-frequency variants are established, the detection of minor variants (<10%) in clinical samples should not warrant treatment adaptation, but encourage a closer monitoring of potential drug resistance. Further research is critical to understanding the impact of minor viral subpopulations on drug susceptibility and clinical outcomes.

LMV is known to have a low genetic barrier to resistance, which is further confirmed by the rapid onset of resistance in this case[35]. Addressing the prevention of drug resistance, Alain and colleagues discuss the possible benefit of therapeutic drug monitoring to verify LMV concentrations in blood, as well as the possible role of standing variation in CMV breakthrough viremia during LMV treatment[36]. LMV does not inhibit viral DNA replication, but prevents the packaging of viral DNA and the maturation of infectious virions[37]. Especially in highly immunocompromised SCID patients, this mechanism of action might allow for low-level viral replication where a mutation could silently emerge, allowing rapid expansion under antiviral pressure.

As the case described here shows, pediatric patients with SCID who develop a severe CMV infection and receive long-term, often complex regimens of antiviral therapy are at a high risk for the development of drug resistance. Resistance screening is crucial to prevent severe outcomes in this vulnerable population, and should be performed when antiviral treatment fails to achieve significant viral load reduction after two weeks. Repeated screening is necessary for a prompt clinical response to emerging resistance, as viral drug susceptibility can be impacted by a rapidly changing composition of the viral population.

Most samples were whole blood, while four of the fifteen samples were CSF. In contrast to the blood samples, no known CMV antiviral resistance mutations were detected in the CSF. The same genetic polymorphisms were carried in both blood and CSF, indicating that the viral populations originated from the same strain. Compartmentalization has previously been described in CMV infection[38–40]. Differences in antiviral drug distribution between body compartments could explain the divergent viral adaptation. The blood-brain barrier (BBB) limits the penetration of certain antivirals, such as CDV and LMV, into the central nervous system[41]. However, herpesvirus infections have been shown to affect the integrity of the BBB and increase permeability, possibly affecting drug distribution[42,43]. The most plausible hypothesis for the compartmentalization between the viral populations in CSF and blood is the existence of a common viral origin, followed by independent viral adaptation in each compartment. Alternatively, suboptimal antiviral concentrations may have promoted the emergence of minor drug-resistant viral subpopulations in the CSF, which then redistributed into the systemic circulation, where positive selection may have driven the expansion of the drug-resistant viral population in the blood. Unfortunately, our data cannot confirm either hypothesis, as we were unable to analyze the low-frequency viral variants in the CSF using NGS due to low viral load.

Although recent studies have investigated the pharmacokinetics and pharmacodynamics of LMV in the pediatric population, many aspects, including the drug distribution within the body, remain poorly understood[23,31,44–46]. A phase 2b study (NCT03940586) investigating the pharmacokinetics, safety and efficacy of LMV in adolescent HSCT recipients showed that the administration of adult LMV doses (480 mg, 240 mg if co-administered with cyclosporin A) to adolescent HSCT recipients resulted in comparable exposures as in the adult population, with a similar efficacy and safety profile[47]. A phase 3 trial studying LMV prophylaxis after HSCT in pediatric patients is currently ongoing (NCT05711667).

Whole-genome sequencing of blood samples demonstrated the stability of the viral genome. No intrapatient variation was seen apart from the mutations conferring drug resistance, considering both whole-genome consensus sequences and minor variants (5–50% frequency) in highly variable genes and genes encoding glycoproteins. Unfortunately, a comparison with the CSF viral population could not be conducted due to an insufficient quantity of DNA to perform whole-genome sequencing.

CMV infection poses a major concern in immunocompromised newborns. There is an urgent need for further research into pharmacokinetics and -dynamics of existing antivirals, as well as alternative treatment options

such as combination therapy and novel antivirals in the pediatric population. This case illustrates the often underestimated, but at times severe burden of CMV infections in the immunocompromised host and emphasizes the importance of awareness, screening, and the need for effective and safe antiviral treatment strategies.

Methods

Ethics

This study was approved by the local Ethics Committee of KU Leuven (S61201).

Prospective resistance analysis: Sanger sequencing

After DNA was extracted from clinical blood and cerebrospinal fluid (CSF) samples (QIAamp DNA Blood Mini kit, Qiagen), Sanger sequencing was performed of the DP (*UL54*), PK (*UL97*), and viral terminase complex (*UL51*, *UL56*, *UL89*) genes, following previously established protocols (ABI3730 sequencer, Thermo Fisher Scientific)[48]. Sequencing reads were mapped to the reference strain Merlin (NCBI GenBank accession NC_006273.2).

Retrospective variant detection: Illumina sequencing

For samples where resistance mutations were discovered during prospective resistance analysis, targeted, amplicon-based NGS of the *UL54* DP and *UL56* terminase subunit genes was performed retrospectively on the MiSeq v2 DNA sequencer (Illumina), as described previously[48]. CLC Genomics Workbench (v21.0.5, Qiagen) was used to map reads to the Merlin reference and identify variants using the Low Frequency Variant Detection tool.

Whole-genome sequencing and data analysis

Whole-genome sequencing was performed as outlined in a previous study, using the SureSelect XT HS library preparation kit with the SureSelect XT CD CMV RNA probe library for target enrichment (Agilent)[49,50]. Multiplexed samples were prepared for sequencing on a MiSeq v2 DNA sequencer (MiSeq Reagent Kit v2, 500 cycles, Illumina).

CLC Genomics Workbench was used to trim and map reads to the Merlin reference strain. Trimming parameters set the quality limit at 0.05 and short (<15 bp) and long (>1000 bp) reads were discarded. Target enrichment was also verified by mapping reads to the human reference genome (GRCh38/hg38). The Low Frequency Variant Detection tool identified variants with a minimum coverage of 15 reads.

Data availability

Targeted Illumina sequencing reads have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject number PRJNA1193001. Whole-genome sequences of CMV samples are available at NCBI GenBank (accession numbers PQ851837- PQ851844).

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Author contributions

Conceptualization, F.H., R.S. and G.A.; Methodology, F.H., S.G., R.S. and G.A.; Validation, F.H., S.G.; Formal analysis, F.H., S.G. and G.A.; Investigation, F.H. and S.G.; Resources, P.C. and P. Mazilier; Data Curation, F.H.; Writing – Original Draft Preparation, F.H.; Writing – Review & Editing, F.H., S.G., P.C., P. Mazilier, P. Maes, R.S. and G.A.; Visualization, F.H.; Supervision, P. Maes, R.S. and G.A.; Project Administration, R.S. and G.A.; Funding Acquisition, R.S. and G.A.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to Graciela Andrei.