Running title: Cytomegalovirus antiviral resistance

Title: Genotypic and phenotypic study of antiviral resistance mutations in refractory cytomegalovirus infection

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ABSTRACT

This study describes the genotypic and phenotypic characterisation of novel human cytomegalovirus (HCMV) genetic variants of a cohort of 94 clinically-resistant HCMV patients. Antiviral-resistant mutations were detected in the *UL97*, *UL54* and *UL56* target genes of 27/94 (28.7%) patients. The genotype-phenotype correlation study resolved the status of 5 uncharacterised UL54 DNA polymerase (G441S, A543V, F460S, R512C, A928T) and 1 UL56 terminase (F345L) mutations found in clinical isolates. A928T conferred high triple-resistance to ganciclovir, foscarnet and cidofovir, and A543V had 10-fold reduced susceptibility to cidofovir. Viral growth assays showed G441S, A543V and F345L impaired viral growth capacities compared with wild-type AD169 HCMV. 3D modelling predicted A543V and A928T phenotypes but not R512C, reinforcing the need for individual characterisation of mutations by recombinant phenotyping. Extending mutation databases is crucial to optimize treatments and to improve the assessment of patients with resistant/refractory HCMV infection.

Keywords: cytomegalovirus, antiviral drugs, resistant mutations, phenotype, genotype.

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BACKGROUND

Characterisation of human cytomegalovirus (HCMV) antiviral drug resistance mutations has contributed to improving HCMV therapy and to the knowledge of viral proteins that serve as new antiviral targets. Ganciclovir (GCV), its oral prodrug valganciclovir (VGCV), foscarnet (FOS) and cidofovir (CDV) target the viral UL54 DNA polymerase and are currently licensed for treatment of HCMV infection [1, 2]. Maribavir (MBV) is an inhibitor of UL97 phosphokinase undergoing a phase 3 clinical trial, however, the FDA has not yet approved MBV for patients with post-transplant HCMV infection who do not respond to the antivirals available [3]. Letermovir (LMV) targets the viral terminase complex (*UL51, UL56, UL89*) and has recently been approved for primary prophylaxis of HCMV infections in allogenic haematopoietic stem-cell transplant (HSCT) recipients [4]. HCMV antiviral resistance is an underestimated emergent problem, especially in transplant recipients, presenting an incidence of 5-12% [5].

Resistance to VGCV/GCV, as first-line therapy, is most commonly due to mutations in *UL97* involved in the initial phosphorylation of GCV [6]. MBV resistance mutations have primarily been mapped to this gene and show compensatory mutations in *UL27* [7]; however, only a few mutations in *UL97* cause MBV-GCV cross-resistance [8, 9]. Mutations in *UL54* are associated with resistance to FOS and CDV and can also appear after prolonged GCV therapy, contributing to a high level of resistance to GCV and inducing cross-resistance to FOS or/and CDV [10]. Resistance mutations are mainly located in the conserved domains of the polymerase, whereas natural polymorphisms appear in the highly variable regions located between domains delta-C and II and between domains III and I [11]. Conversely, LMV resistance mutations are mainly described in the *UL56* terminase subunit and rarely in *UL89* and *UL51* [12].

Confirmation of antiviral resistance is based upon the detection of viral mutations that confer drug resistance by genotypic antiviral resistance testing, providing timely data to facilitate clinical decision making. However, the accuracy of genotypic antiviral resistance testing depends

on validated databases linking specific mutations with levels of drug resistance. 3D protein modelling provides useful prediction of crucial residues for protein-antiviral molecules interaction [13]. Nevertheless, recombinant phenotyping is the reference method to confirm the level of antiviral resistance and the impact of individual mutations on viral growth.

This study describes the incidence of mutations under GCV, FOS, CDV, LMV and MBV therapy in patients with refractory HCMV infection. We also aimed to phenotype previously uncharacterised HCMV genetic variants detected by genotypic methods by 3D protein modelling and recombinant phenotyping.

MATERIALS AND METHODS

Study specimens and population

Positive samples from patients with refractory HCMV infection (n=94) who fulfilled criteria of suspicion of resistance to antiviral treatment (VGCV/GCV, FOS, CDV, LMV, MBV) were included from April 2012 until September 2021 [1]. Antiviral treatment was administered according to the clinical judgement of the attending physician. The study population has been enlarged from a cohort previously published for a different propose [14]. The patients belonged to the hospitals included in the Spanish Network for Research in Infectious Diseases (REIPI) and the Group for the Study of Infection in Transplantation (GESITRA). All samples were collected on suspicion of antiviral resistance, frozen at -80ºC and sent to the coordinating centre (Hospital Clinic of Barcelona, Spain) for performing genotypic antiviral resistance testing.

HCMV load quantification

The HCMV load was measured in liquid samples by quantitative real time polymerase chain reaction (qPCR) in a Cobas 6800 (Roche, Switzerland) according to the manufacturer's instructions. Viral load of gastrointestinal biopsies was quantified by qPCR (Q-CMV Real Time Complete Kit; Nanogen Advanced Diagnostics, Buttigliera, Italy) using a 7300 Real Time PCR System (Applied Biosystems).

Genotypic antiviral resistance testing

Extraction of total nucleic acids from liquid samples was performed in MagNA Pure Compact (Roche, Switzerland), and with the EZ1 DNA Tissue Kit (Qiagen, Hilden, Germany) in paraffin-embedded tissue and fresh tissue according to the manufacturer's specifications and using the Bio-Robot EZ1 (Qiagen).

Genotypic testing was done by Sanger sequencing based on PCR amplification of HCMV *UL97* (residues 270–670), *UL54* (300-1000) and *UL56* (180-395) regions. These regions correspond to resistance-associated domains and were sequenced using previously described primers and procedures [14, 15]. Each amplicon was bidirectionally sequenced to avoid artifacts. Sequences were analysed and aligned using the MEGA v.7. software [16] and were compared with the HCMV TB40 strain [GenBank: MF871618.1] using the MRA-Mutation Resistance Analyzer tool provided by the University of Ulm [17].

Prioritization of sequence variants for phenotyping

The gene position of the variants found was used to establish priority for phenotyping. Mutations located at the two previously characterised hyper-variable non-conserved regions located at residues 614-697 and 874-898 of *UL54* [10] are shown in Table S1 and did not undergo phenotyping.

Phenotypic assay by recombinant bacterial artificial chromosome technology

Mutations with previously uncharacterised phenotypes at the time of genotypic detection were individually tested at the French National Reference Centre for Herpesviruses (Limoges, France) and the Hospital Clinic of Barcelona (Barcelona, Spain) using a phenotypic assay with recombinant bacterial artificial chromosome (BAC) technology as described previously [18]. Each mutation was introduced by "en passant" mutagenesis into a HCMV BAC [19] containing an enhanced green fluorescent protein (EGFP) gene in the unique short region derived from the AD169 laboratory strain (provided by M. Messerle). The recombinant BAC was transfected into

MRC-5 cells (bioMérieux, Lyon, France) using the liposomal reagent Transfast (Promega, Madison, Wisconsin) following the manufacturer's instructions. The presence of the desired mutation was confirmed by Sanger sequencing.

A focus reduction assay in a 48-well MRC-5 culture plate with a multiplicity of infection (MOI) of 0.01 was used to assess antiviral susceptibility in triplicate to GCV, FOS, CDV, and/or LMV according to the treatment received by the patient. The half maximal effective concentration (EC_{50}) of the mutant was compared to that obtained for the wild-type control HCMV BAC.

To estimate the impact of each mutation on viral growth, the recombinant strain and the AD169-EGFP control were inoculated into 48-well MRC-5 culture with an MOI of 0.01. The number of fluorescent plaque-forming units (PFU) was counted from days 1 to 4 and on day 7 post-inoculation to establish viral growth curves for each recombinant.

Structure of the protein 3D model

The theoretical structure of the UL54 DNA polymerase was built by homology modelling with the standalone version of MODELLER 9.9 [20]. The UL54 sequence was aligned with three templates as described previously [21]. Sequence alignment included primary structures of the UL52 homolog from herpes simplex virus-1 (HSV-1) (PDB: 2GV9), the C-terminal part of UL54 taken from PDB: 1YYP (i.e., complex of UL44 with fragment 1223-1242 of UL54), and the exonuclease domain (i.e. amino acid sequence 109-342), metal ions and DNA duplex of PDB: 1CLQ. UL54 moieties aligned to non-resolved loops of UL52 were retrieved from sequence alignment for calculations. A fast molecular dynamic optimisation implemented in MODELLER was applied to each 100 calculated structures. Quality structures were assessed by calculating their Q-mean score on a dedicated web server [22]. For *UL56* mutations, the model with an herpes simplex virus-1 homolog did not allow localising the mutations concerned as there was no correspondence with the amino acid sequence.

Ethical approval

This study was approved by the Ethical Committee of the Hospital Clínic of Barcelona (ref. nº HCB/2018/0634) as the reference committee for all the participating hospitals endorsed by GESITRA according to CPMP/ICH/135/95 regulations. All the patients included in the study provided signed informed consent.

RESULTS

Overview of *UL54*, *UL56* and *UL97* sequence variation under treatment

This study comprised a cohort of 94 patients with refractory HCMV infection who fulfilled the criteria of suspicion of HCMV resistance to standard antiviral treatment (GCV, FOS, CDV). Seven patients additionally received LMV and 4 MBV as salvage therapy. One clinical sample from each patient was collected at the time of suspicion to perform genotypic antiviral resistance testing. Subjects with previously characterised mutations associated with either resistance or sensitive response to antivirals located in the target *UL97, UL54* and *UL56* genes were classified according to their clinical history and sample type (table 1).

Previously described resistant mutations were detected in 24/94 (25.5%) patients, all of whom were transplant recipients (6 HSCT, 18 solid organ transplant (SOT)): 19 patients had resistant mutations in *UL97* (one presenting two mutations), 2 in *UL54* (one presenting two mutations), 1 patient with mutations in *UL54* and *UL56,* and 2 in *UL54* and *UL97*. These mutations conferred resistance to GCV, FOS, CDV, LMV or MBV, as well as multiple- or cross-resistance (Table 2). Data of cumulative treatment, time from transplantation until the detection of the variant, viral loads of the detection sample and level of resistance to each antiviral are shown in Table 2.

The incidence of natural polymorphisms, previously described to be sensitive to antivirals, was 76/94 (80.9%): 5 (5.3%) patients presented them in *UL97*, 67 (71.3%) in *UL54* and 4 (4.3%) in *UL56* (table 1, table S1).

Fourteen previously undescribed genetic variants were found in 16/94 (17.0%) patients, all located in *UL54* but 1 in *UL56* after LMV therapy (table 3, table S1). Each genetic variant was detected in one patient, except for *UL54* A543V and S883I that were detected in 2 different individuals. Five *UL54* and 1 *UL56* variants located outside hypervariable regions were selected for recombinant phenotyping and 3D protein modelling. The *UL56* P800L substitution was previously described by Champier *et al*. in one LMV-naïve patient [23]. However, as it was detected in association with a LMV resistance mutation in this study, we wanted to measure its potential impact on decreasing sensitivity to LMV and on viral replicative capacity.

Phenotypic results

For antiviral drug susceptibility assays, the resistance index (RI) of each mutant was calculated by dividing the EC_{50} value (µM) of the mutant strain by the EC_{50} of the AD169 HCMV control strain (Table 3). Replicative capacity assays were performed for each mutant and in parallel with the AD169 for each repetition (Table 3; Figure 1).

UL54 A928T presented a triple-resistant pattern to GCV, FOS, CDV at a high level of resistance that any antiviral concentration could inhibit 50% of the replication of the A928T mutant. *UL54* A543V conferred 10-fold decreased susceptibility to CDV, and impaired growth capacity. The three remaining *UL54* (G441S, F460S, R512C) were sensitive to GCV, FOS and CDV, but G441S involved a defective replicative capacity (Figure 1).

None of the phenotypes of the *UL56* mutations (F345L, P800L) conferred reduced response to LMV, or cross-resistance to the DNA polymerase inhibitors tested. However, both mutations involved lower viral kinetics of replication (Figure 1).

Correlation of newly characterised phenotype mutations with clinical outcomes

UL54 A543V was detected in two kidney transplant recipients in combination with the known GCV-resistant mutation *UL97* M460I (figure 2A). In one patient, this mutation emerged after 90 days of GCV and 9 days of FOS treatment, reaching viral loads of 137000 IU/ml. In the other

patient, the mutation was detected after 37 days of prophylaxis with VGCV, with a viral load of 3100 IU/ml. The phenotypic results showed A543V conferred a defective replicative capacity and resistance to CDV (table 3), which was not administered to either subject.

UL54 A928T was detected in a plasma sample with a viral load of 21732 IU/ml in a congenital HCMV individual after 2 HSCT (145 days after the first intervention, and 11 after the second) (figure 2B). This mutation was detected after cumulative treatment of 43 days of GCV and 23 days of FOS. CDV was administered afterwards because of adenovirus reactivation and was maintained until limitation of therapeutic efforts; however, HCMV DNA clearance was not achieved until its death. This clinical unresponsiveness correlated with the *in vitro* phenotypic results of GCV, CDV and FOS multi-resistance.

A *UL56* F345L missense mutation was located within the LMV-resistance region (residues 230-370) (Figure S2) in a HSCT recipient after 209 days of VGCV/GCV and 20 days of LMV but not before LMV therapy. Viral loads were 3 $\log_{10}$ IU/ml and were well-controlled until HCMV DNA cleared after 148 days with LMV (figure 2C). This mutation conferred a highly impaired replicative capacity and was sensitive to GCV, FOS, CDV and LMV in concordance with the clinical antiviral response and clearance after the detection of F345L (Table 3).

P800L was detected in an HSCT recipient at day 163 post-transplant, 7 days before LMV therapy onset (figure 2D). After 28 days receiving LMV, the high level LMV-resistant mutation C325F emerged together with P800L. Despite discontinuation of LMV, both mutations persisted until the patient developed multiorgan failure.

Follow-up of viral loads and antiviral treatment could not be retrieved for the subject with the *UL54* G441S variant. The clinical data of patients infected with wild-type phenotypes characterised in this study are not presented.

Predicting the phenotype of new UL54 variants by 3D protein modelling

UL54 3D protein modelling showed that the amino acids G441S (figure 3A) and F460S (figure 3C) were localised in non-structured regions of UL54 DNA polymerase. These domains are outside conserved regions. This agrees with the absence of impact on antiviral resistance obtained by phenotypic methods. R512C (figure 3D) and A543V (figure 3B) are part of a helix. Removing one amino acid of a helix can change repartition of amino acids exposed to solvent or the interior of the protein. Both are localised in the Exo-III/delta-C region but are associated with a completely different impact on antiviral resistance as shown by recombinant phenotyping. A928T (figure 3E, figure S1) is part of a helix between region I and VII of the palm domain. The change of an alanine to a threonine impacts both the polarity and the size of the amino acid, modifying the potential interaction between *UL54* and antivirals.

DISCUSSION

The results of this study demonstrate a high incidence (n=27/94, 28.7%) of antiviral-resistant mutations in patients with refractory HCMV infection, which is even higher considering all of them emerged in transplant recipients (n=27/83, 32.5%). Recombinant phenotyping of previously uncharacterised mutations showed that the *UL54* A928T mutation conferred high level resistance to GCV, FOS and CDV, and *UL54* A543V conferred intermediate-resistance to CDV. Additionally, *UL54* G441S, A543V and *UL56* F345L, P800L variants showed lower growth capacities than wild-type AD169 HCMV.

Uncharacterised mutations were selected for the correlation of their gene position to antiviral resistance. G441S and F460S were located in the region associated with GCV and CDV cross-resistance (figure S2), but neither conferred resistance to these antivirals, as predicted *in silico.* In agreement, the F460L variant, located in the same residue, was described to be sensitive (1.2-fold shift) to DNA-polymerase inhibitors and did not show a slow-growth phenotype [4].

A543V and R512C were mutations located in the Exo-III/delta-C domain. This domain has been described as being involved in exonuclease and polymerase catalytic function, which are well-conserved among mammals and yeast, and was associated with resistance to GCV, FOS and CDV (figure S1) [24]. Our study showed A543V conferred CDV resistance and impaired its viral growth, whereas a previously reported A543S mutation had a wild-type phenotype [4], suggesting that when alanine is substituted by valine, its bulky lateral aliphatic chain could block its polymerase function, but the small hydroxyl group of the serine does not. Therefore, residue A543 seems to play an important role in viral growth. However, this variant clearly replicated in the two clinical cases presented (Figure 2A), suggesting an A543V growth defect could be compensated by other mutations, such as *UL97* M460I.

The A928T mutation located just outside the catalytic polymerase function, in a region supposedly not associated with a lack of antiviral response, conferred high resistance to three DNA polymerase inhibitors (figure S1). Previous reports showed certain triple-resistance mutations in the surroundings, such as A836P and del981-2, detected in patients receiving prolonged therapy and causing loss of growth fitness [34]. Conversely, many variants in the nearby amino acids (V902G, E903G, K947E, M959T) were susceptible to GCV, FOS and CDV [4]. This results also strengthens the importance of discerning high from intermediate-low levels of resistance, as this influences the dosages and antiviral molecules chosen in clinical practice.

UL56 mutations emerged in the context of infections unresponsive to standard therapy and the use of LMV as salvage therapy. F345L was located inside the LMV resistance region (figure S2) and strongly impaired viral replication, but surprisingly did not involve a loss of response to the different antiviral molecules tested. This suggests that the F345 residue is critical for biological terminase function but not for interaction with LMV, as has been described for R215, since R215C amino acid substitution conferred advanced growth capacity but not loss of response to LMV [14].

297 The P800L polymorphism emerged before LMV therapy and remained detectable until the
298 patient's death after 28 days of LMV in association with the C325F resistance mutation (figure
299 1D), suggesting the need to measure its potential impact on decreasing sensitivity to LMV and
300 on viral replicative capacity. Phenotypic assays showed this mutation impaired virus growth and
301 was confirmed as having no effect on virus sensitivity to LMV. As the P800L+C345F variant
302 continued replicating, the defective growth associated with P800L alone seemed to be
303 compensated by the emergence of C325F, the residue of which appeared to be critical for LMV
304 binding but unimportant for HCMV terminase function.

305 It has been described that *UL54* mutations can confer multi-resistance and may emerge after a
306 previous *UL97* mutation, enhancing the level of resistance [18], as shown in 4 SOT recipients in
307 our cohort. Nevertheless, this study presents the uncommon detection of 2 *UL54* resistance
308 mutations with no previous change in *UL97* and the combination of mutations in *UL54* together
309 with *UL56* after GCV+LMV therapy.

310 Fewer natural polymorphisms arose in *UL97* than in *UL54* during standard treatment, supporting
311 the existing literature [6, 11], and hardly any were detected in *UL56,* in agreement with the
312 previously described *UL56* genetic conservation among herpesvirus before LMV therapy [14,
313 35]. However, the prevalence of natural polymorphisms in *UL56* and *UL97* was understated as
314 full-length genes were not amplified and only the regions associated with drug-resistance were
315 sequenced.

316 This study was limited by the incapacity to recover previous isolates of the patients infected with
317 the newly characterised variants in order to determine the timing of the earliest emergence of
318 the respective mutations. Phenotyping assays involve long-laborious work and results were
319 obtained months after the genotypic resistance testing was requested.

320 3D protein modelling is a useful and fast tool to predict the impact of genetic variants *in silico*.
321 It correctly predicted the effect of A928T, A543V, G441S and F460S mutations on antiviral

susceptibility, but did not with the R512C mutation, highlighting its limits and the need for confirmation by recombinant phenotyping. Therefore, the remaining genetic variants located in the *UL54* highly variable regions detected in this study require future recombinant phenotyping.

This study reinforces the fact that genotype-phenotype correlation studies not only serve to define the level of antiviral resistance and viral kinetics associated with each genetic variant, but also determine the biological role of each residue and allow constructing comprehensive and reliable mutation maps to help develop new anti-HCMV therapies. The high incidence of HCMV resistance mutations in transplant recipients presents a worrisome scenario for their clinical management. However, early genotypic testing and increasing databases of mutations by phenotyping can optimise treatments and improve the assessment of patients with refractory/resistant HCMV infection.

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Potential conflict of interest

None of the authors report any conflict of interest. All authors have submitted the ICMJE Form for Disclosure of Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Figure legends

Figure 1. Growth capacity of newly characterized HCMV variants. Each recombinant cytomegalovirus (CMV) strain was compared with an AD169 CMV control strain. Each strain was inoculated at an equal multiplicity of infection of 0.01. Plaque-forming units (PFUs) were counted from days 1 to 4 and on day 7 post-inoculation. Data shown are the mean of 3 replicates set up simultaneously, with the corresponding standard deviation. The AD169 curve is the mean of 7 different experiments (one repetition per variant).

Figure 2. Clinical follow-up of patients with newly characterised phenotype mutations. Viral loads in IU/mL were tracked against days after transplantation. Anti-HCMV treatment outset and end are indicated. Genotypic assays were performed in the clinical samples indicated with an arrow. New phenotype mutations in *UL54* (A-C) and *UL56* (C-D) are indicated in bold, together with previously characterised resistant mutations. Abbreviations: VGCV valganciclovir; GCV ganciclovir; LMV letermovir; FOS foscarnet; CDV cidofovir.

Figure 3. 3D protein models showing the location of newly characterised *UL54* mutations. Theoretical structures of UL54 calculated with MODELLER are represented in cartoon mode. The different domains close to the new mutations are coloured as follows: residues 379 to 421 of region IV/Exo-II in orange, residues 492-588 of region delta-C/Exo-III in yellow, residues 905 to 919 of region I in blue and residues 962 to 970 of region VII in grey. Purple dots in active sites stand represent metal ions. The DNA duplex is coloured in orange and blue. (A) The G441S mutation in red between the Exo-II and Exo-III regions. (B) The A543V mutation in red in the Exo-III region. (C) The F460V mutation in red between the Exo-II and the Exo-III regions. (D) The R512C mutation in red in the Exo-III region. (E) The A928T mutation in red between regions I and VII.

TABLES

Table 1. Clinical history of the study population. Human cytomegalovirus viral loads (IU/ml) and number of subjects with resistance mutations and polymorphisms in the target genes are indicated according to immunosuppression and the type of sample.

Clinical		**Resistance mutation**			**Sensitive polymorphisms**		
History	**N[a]**	***UL97***	***UL54***	***UL56***	***UL97***	***UL54***	***UL56***
Congenital HCMV	6	0	0	0	0	5	0
HIV	2	0	0	0	1	2	0
IBD	2	0	0	0	0	1	0
CVID	1	0	0	0	0	1	0
Transplant recipients	**83**	**22**	**5**	**1**	**4**	**58**	**3**
HSCT	30	3	3	1	2	23	2
SOT	53	19	2	0	2	35	1
● Heart	11	4	1	0	0	5	1
● Lung	6	4	1	0	0	5	0
● Liver	8	5	0	0	0	6	0
● Kidney	25	5	0	0	2	17	0
● Liver-kidney	1	0	0	0	0	1	0
● Pancreas-kidney	2	0	0	0	0	1	0
Sample type							
● Plasma	84	21	3	1	5	58	3

• whole blood	4	1	1	0	0	4	0
• GI biopsy	5	0	0	0	0	4	0
• Aqueous humour	1	0	0	0	0	1	0
Total	**94**	**22**	**5**	**1**	**5**	**67**	**3**

N[a]: indicates the number of subjects.

Abbreviations: SNP Single Nucleotide Polymorphism, HCMV Human Cytomegalovirus, HIV Human Immunodeficiency Virus, IBD Inflammatory Bowel Disease, CVID Common Variable Immunodeficiency, HSCT haematopoietic stem cell transplant, SOT solid organ transplant, GI Gastrointestinal.

Table 2. Resistance mutations detected in association with current antiviral drugs in the overall study population.

Gene	Mutation	N[a]	Transplant[a]	Days of cumulative treatment[a,b]	Days until detection[c]	Viral load at the detection (IU/ml)[d]	Resistance level[e]	Reference
UL97	M460V	2	HSCT, Liver	19d VGCV/GCV (n=2)	493	5,82E+03	5-10x GCV	[24]
UL97	M460I	2	Kidney (n=2)	69d VGCV (n=2)	184	7,01E+04	5-10x GCV	[25]
UL97	C480F	2	HSCT, Kidney	60d MBV (n=2), 67 GCV (n =2)	222	9,97E+03	2-5x GCV, 223x MBV	[8, 9]
UL97	C592G	1	Kidney	138d VGCV	235	9,87E+03	2-5x GCV, 2-5x FCV	[26]
UL97	A594V	5	Kidney (n=2), liver (n=2), lung	56d (14; 121) VGCV/GCV (n=5), 52d FOS + 51d IgG (n=1)	137 (100; 156)	2,03E+04 (1,20E+04; 3,68E+04)	5-10x GCV	[27]
UL97	A594P	1	Heart	24d GCV	534	1,84E+04	5-20x GCV	[26]
UL97	L595S	5	HSCT, heart, lung (n=2), kidney	38d (20; 54) VGCV/GCV (n=5), 36d FOS + 16d IgG (n=1)	158 (66; 357)	3,87E+04 (2,29E+04; 5,80E+04)	5x GCV	[28]
UL97	L595W + A594V	1	Heart	14d VGCV	110	4,12E+03	5.1x GCV	[28]
UL54	V781I	1	HSCT	38d GCV + 79d FOS + LT	153	1,63E+03	1-4x GCV, 4-5.2x FOS	[29]

UL54	**A928T**	1	cCMV + 2HSCT	43d GCV + 23d FOS	145	2,17E+04	NP	
UL54	L773V + G841A	1	HSCT	35d VGCV/GCV + 45d FOS + 10d ACV	133	3,55E+03	2x GCV, 5x FOS / 3.2x GCV, 2.6x CDV, 4.3x FOS	[30, 31]
UL54 + UL97	D413N + M460I	1	Lung	24d GCV	668	6,20E+04	6.5x GCV, 11x CDV	[32]
UL54 + UL97	**A543V** + M460I	2	Kidney (n=2)	64d VGCV/GCV + 9d FOS	185	7,01E+04	NP /5-10x GCV	[25]
UL54 + UL97	A987G + C603W	1	Heart	150d VGCV prophylaxis + 84d VGCV	238	1,90E+04	6.8x GCV, 5.3x CDV / 8.3x GCV	[33, 29]
UL54 + UL56	T700A + C325F	1	HSCT	33d VGCV/GCV + 74d FOS + 4d CDV + 27d LMV + LT	198	3,41E+03	4.7x FOS / >3000x LMV	[31, 34]

[a] N: number of patients infected with the HCMV mutant indicated, receiving the transplant type, receiving the treatment indicated.

[b] Days of cumulative treatment until the time of mutation detection are indicated as the median and the (Q1; Q3) when n>2

[c] Days until detection are calculated from the transplant date until the detection of the variant in the clinical sample by sequencing are indicated as the median (Q1; Q3)

[d] Viral loads of the clinical sample in which the variant was detected are indicated as the median (Q1; Q3) in IU/ml.

[e] Level of resistance is indicated as fold-shift reduction of the effective concentration 50% (EC50) of the mutant compared with the CMV control strain.

New phenotype mutation in this study are indicated in bold and by NP (new phenotype) resistance level.

Abbreviations: HSCT hematopoietic stem cell transplant, cCMV congenital cytomegalovirus infection, VGCV/GCV valganciclovir/ganciclovir, MBV maribavir, FOS foscarnet, CDV cidofovir, LMV letermovir, ACV acyclovir, FCV faldaprevir, IgG CMV-specific Immunoglobulin G, LT CMV-specific lymphocytes T infusion.

Table 3. Results of antiviral susceptibility and replicative capacity assays of novel HCMV genetic variants.

Gene	Mutation	N[a]	Patient history	Antiviral susceptibility (RI)[b]			
				GCV	CDV	FOS	LMV
UL54	G441S	1	CVID	0.67 (± 0.24)	1 (± 0.75)	1.13 (± 0.8)	
UL54	F460S	1	HSCT	0.86 (± 0.45)	0.88 (± 0.78)	1.11 (± 0.8)	
UL54	A543V	2	Kidney (2)	1.5 (± 0.1)	**10 (± 5.37)**	1.21 (± 0.27)	
UL54	R512C	1	Kidney	1	1	0.74	
UL54	A928T	1	cCMV + 2 HSCT	**>3**	**>3**	**>3**	
UL56	F345L	1	HSCT	1.42 (± 1.07)	1.67 (± 46)	0.98 (± 0.04)	0.99 (± 0.04)
UL56	P800L	1	HSCT	0.23	0.13	1	1.11

N[a]: number of patients in whom the mutation was detected.

RI[b]: The resistance index is the EC50 value (effective concentration 50% (µM)) for the mutant strain divided by the EC50 of the AD169 HCMV control strain. Data are indicated as the mean of 3 biological repetitions of 3 independent experiments and standard deviations (except for R512C, P800L which involved 3 biological repetitions of a single experiment)

RI >3 is considered drug resistant and is indicated in bold.

Abbreviations: HSCT haematopoietic stem cell transplant, CVID common variable immunodeficiency, GCV ganciclovir, CDV cidofovir, FOS foscarnet, LMV letermovir.

Figure

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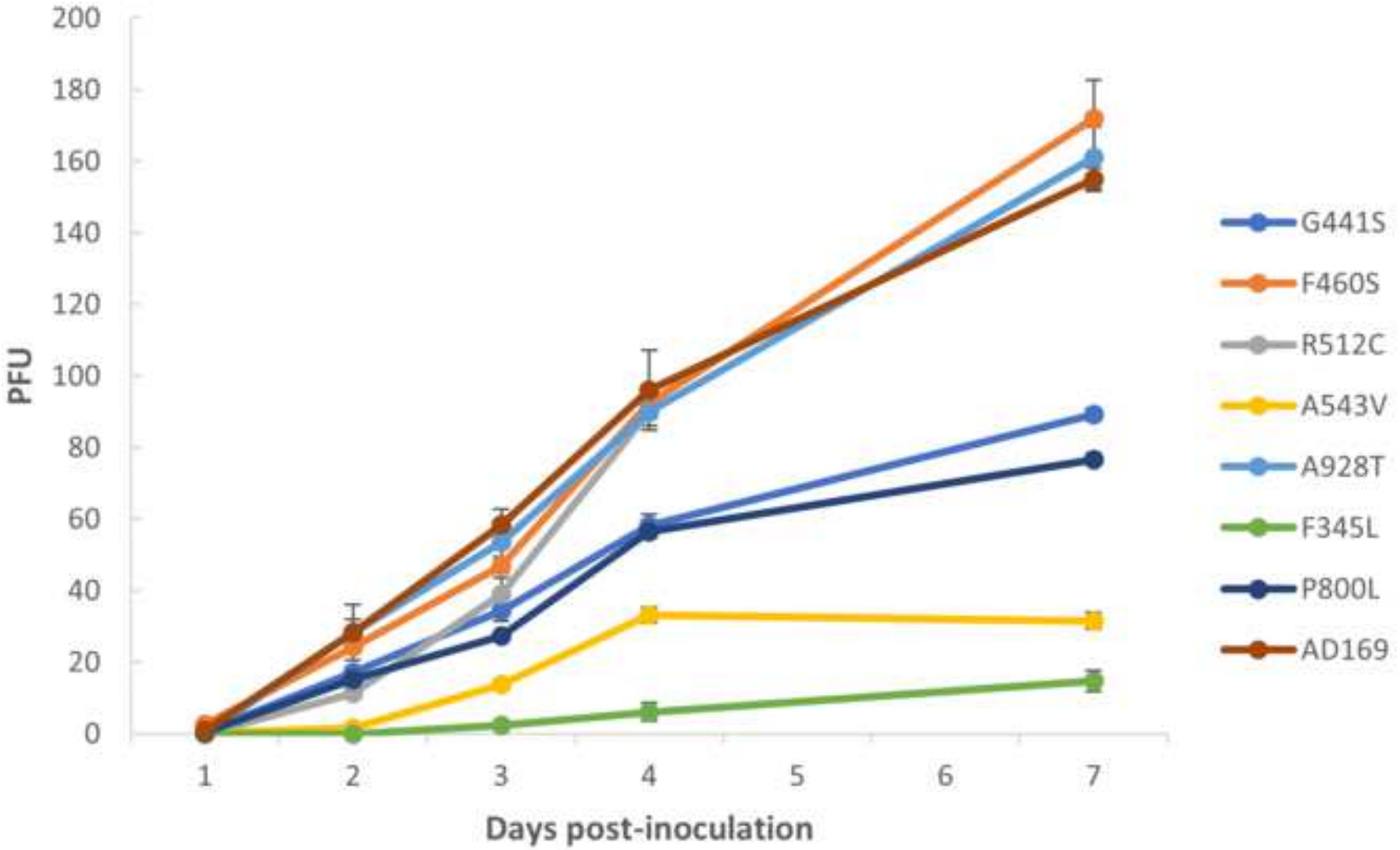

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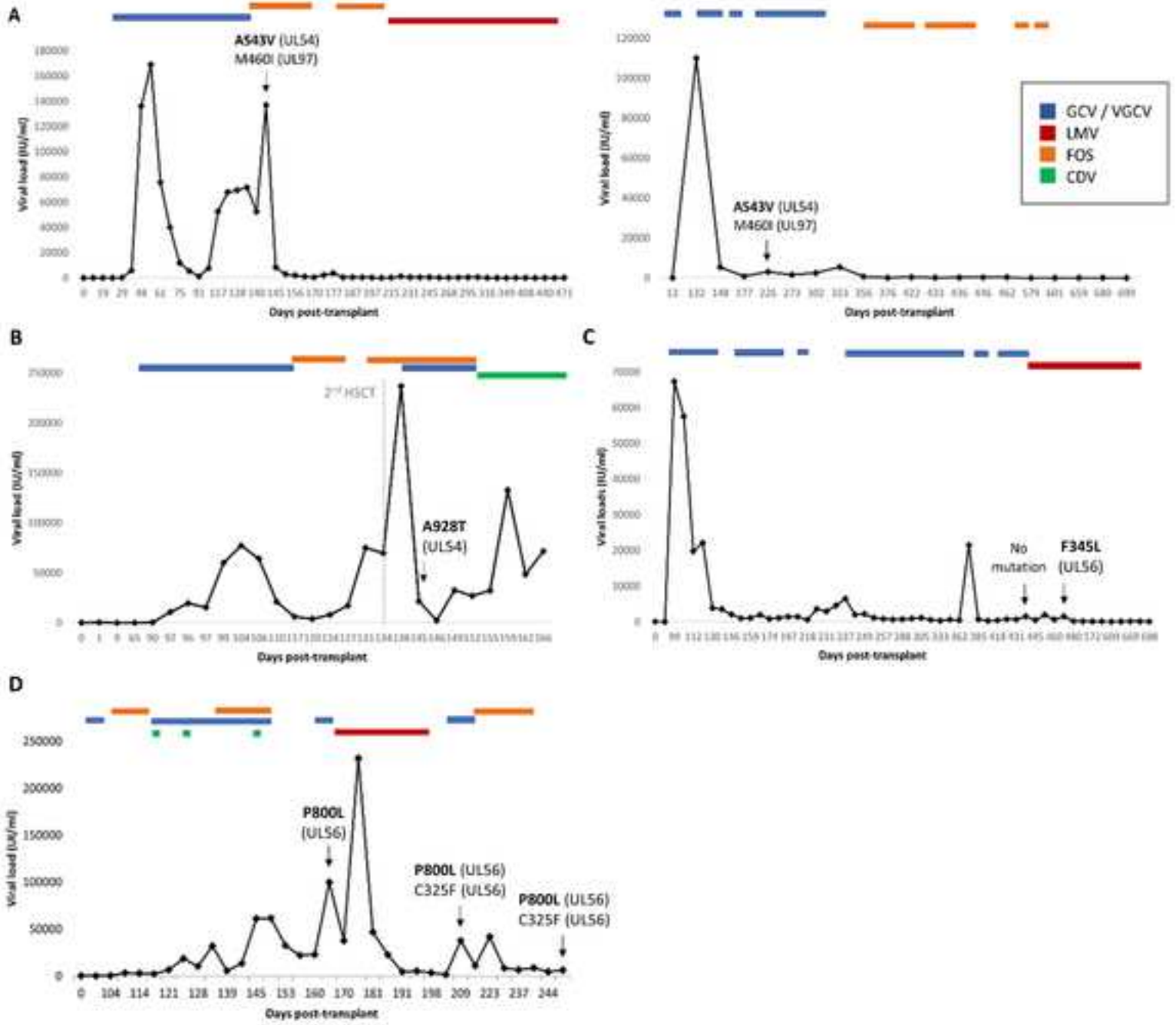

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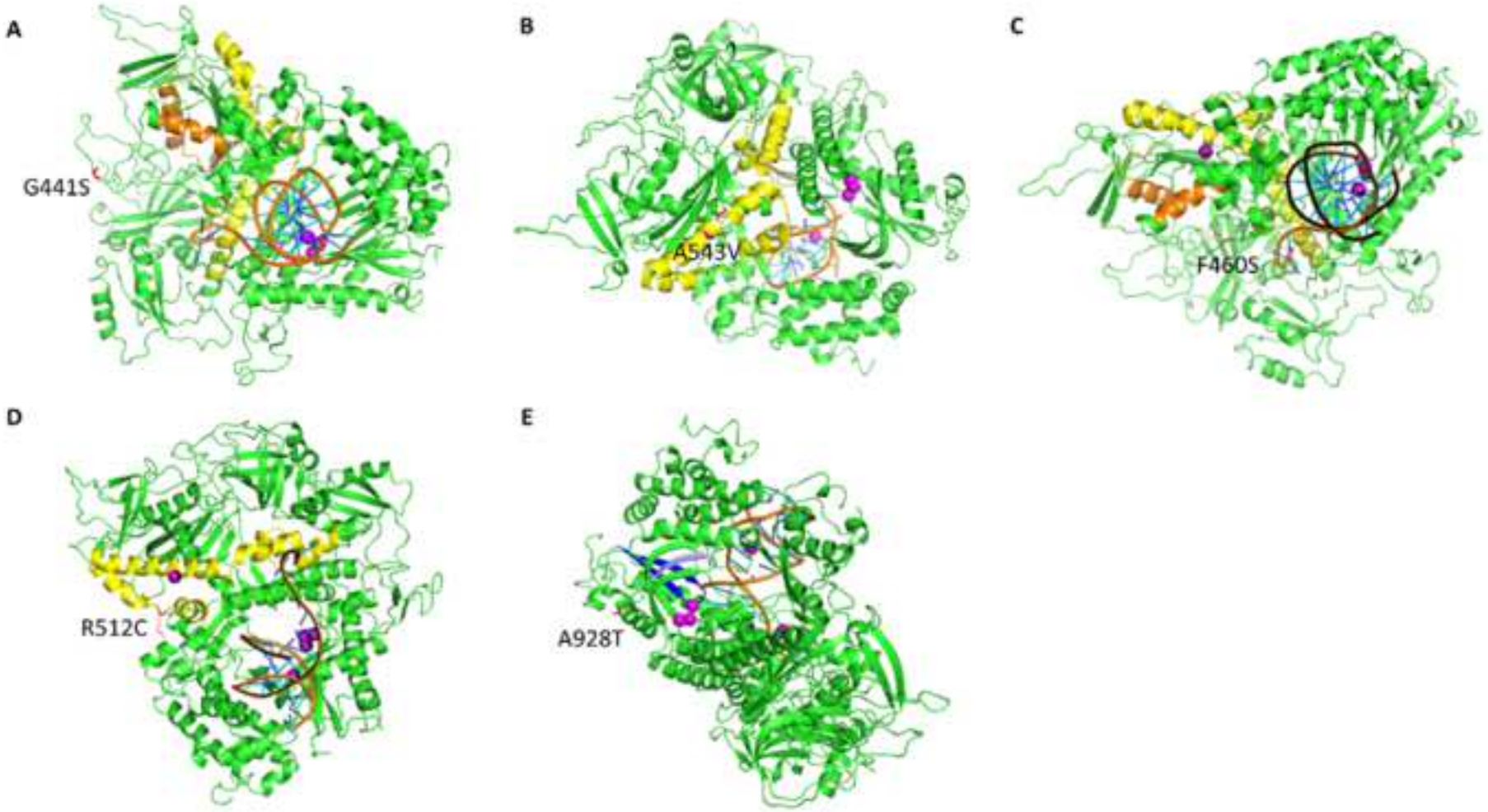

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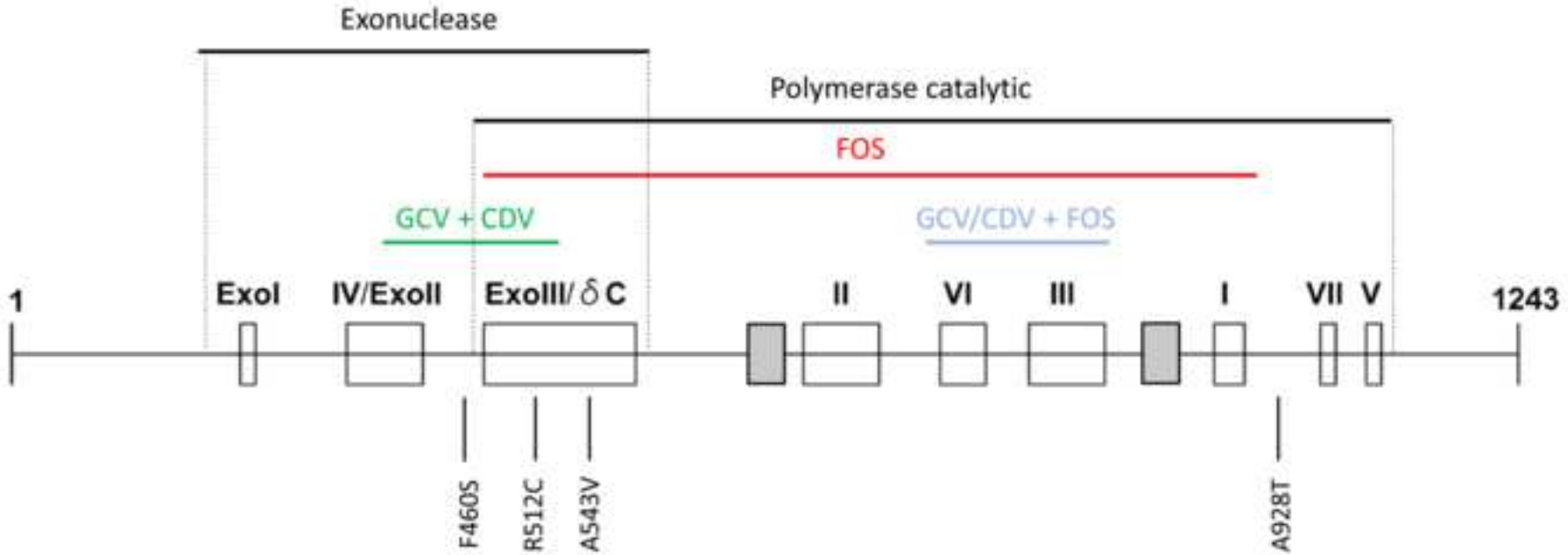

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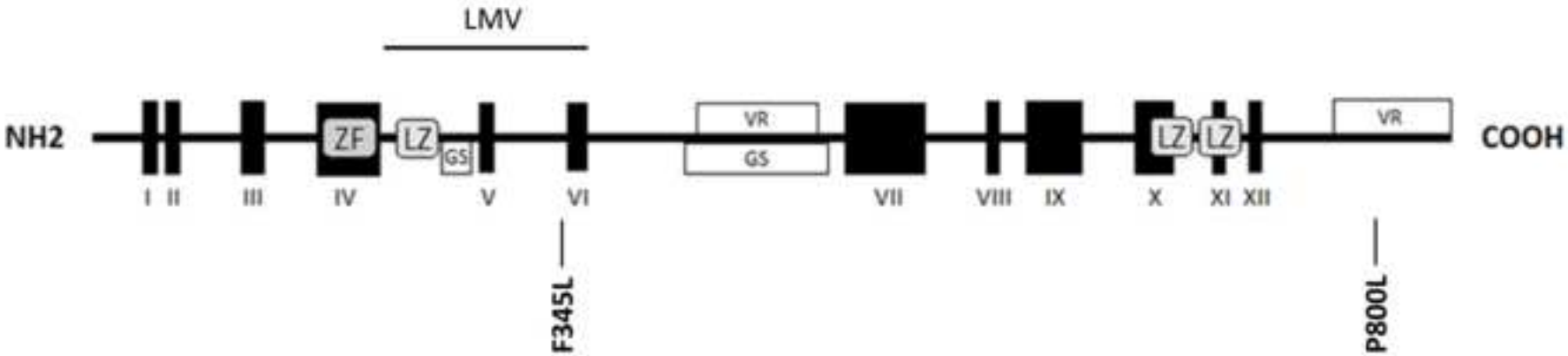

SUPPLEMENTARY DATA

Table S1. Genetic variants detected by Sanger sequencing.

Sensible polymorphisms			**Uncharacterised variants**		
Gene	**Mutation**	**N**	**Gene**	**Mutation**	**N**
UL97	N510S	2	UL54	V615S	1
UL97	V758M	1	UL54	Q639H	1
UL54	S612N	1	UL54	G657D	1
UL54	L655S	35	UL54	S682F	1
UL54	S685N	35	UL54	G687S	1
UL54	A688V	1	UL54	S883I	2
UL54	A692V	1	UL54	E888K	1
UL54	F699L	10	UL54	G889E	1
UL54	V759M	1			
UL54	S771P	1			
UL54	I837V	2			
UL54	G841A	1			
UL54	G874R	1			
UL54	T885A	21			
UL54	Ins885S	3			
UL54	P887S	1			
UL54	L890F	1			
UL54	S895N	1			
UL54	S897L	10			
UL54	D898N	28			
UL56	S227R	1			
UL56	R246C	2			

N: number of patients in whom the variant was observed.

Figure legends

Figure S1. Scheme of the CMV *UL54* gene with novel mutations. Conserved regions are indicated in white boxes (domains I-VII, delta-C); hypervariable regions are indicated in grey boxes. Ranges of codons containing drug resistance mutations to ganciclovir (GCV), foscarnet (FOS) and cidofovir (CDV) are specified. Novel mutations are located between IV-deltaC, within the delta-C domain and between regions I-VII. This gene map is described according to [1].

Figure S2. Scheme of the protein UL56 domain organisation with the novel phenotype mutations. Conserved regions are indicated in black boxes (domains I-XII); variable regions (VR) and glycine and serine-rich flexible region (GS) in white boxes; leucine zippers (LZ) and zinc finger domain (ZF), the metal-binding site of which is located in region IV. The region containing mutations associated with resistance to letermovir is indicated [2, 3]. The two novel mutations are located adjacent to domain VI and inside variable region (residues 778-850). The *UL56* genetic structure is based on the previously described scheme [4].

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