Characterization of Cytomegalovirus Resistant Strains in Hematopoietic Stem Cell Transplanted Patients

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Preface

This study was realized in the Molecular Oncology & Viral Pathology Group of the Portuguese Oncology Institute of Porto (IPO Porto) with the collaboration of the Faculty of Medicine of the University of Porto.

A review article was submitted to publication in *Reviews in Medical Virology*: Campos AB., Ribeiro J., Bouttoleau D., Sousa H., **Human Cytomegalovirus Drug-Resistance Mutations in Stem Cell Transplantation: Current State of the Art**.

The results obtained in this study will be submitted to publication: Campos AB., Ribeiro J, Bouttoleau D, Sousa H., **Characterization of Cytomegalovívus strains in Hematopoietic Stem Cell Transplanted Patients in Portugal**.

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Abstract

Human cytomegalovirus (HCMV) infection is recognized as the major cause of morbidity and mortality in patients who receive hematopoietic stem cell transplant (HSCT). Currently, four antiviral drugs, Ganciclovir, Valganciclovir, Foscarnet and Cidofovir are used to prevent and treat HCMV infection and diseases. However, prolonged antiviral therapy, which is often necessary, may lead to antiviral resistance associated with the development of mutations in the phosphokinase (*UL97*) and/or DNA polymerase (*UL54*) viral genes. The characterization of HCMV drug resistance mutations is an important issue, improving the management of patients with alternative treatments. This study aims to characterize HCMV *UL97* and *UL54* mutations in twenty two patients with HCMV infection submitted to allogeneic HSCT at the Portuguese Oncology Institute of Porto (IPO Porto). To characterize HCMV mutations, DNA fragments of both *UL97* and *UL54* were amplified by nested PCR and sequenced for genetic characterization. The genetic information was compared with the reference HCMV strains and described as resistance mutations, polymorphisms or unknown mutations. Resistance mutations were identified in seven patients (32%): five (23%) harbored HCMV resistance mutations in *UL97* (C592G, A594V, L595W and C603W) and two (9%) harbored HCMV resistance mutations in *UL54* (P522S and L957F). These *UL97* resistance mutations are amongst the most frequently detected mutations in HCMV. *UL54* gene was less frequently mutated and its mutations were detected in the absence of *UL97* mutations. The P522S mutation is one of the most frequent *UL54* gene mutations while L957F mutation has only been reported on laboratory strains, until now. Several polymorphisms were also found in both genes in combination with the resistance mutations. Unknown mutations in *UL97* and/or in *UL54* were also found, which may play an important role in the emergence of antiviral resistance. Resistance mutations, which lead to an impaired response to the therapy, confer different resistance levels which are an important factor that influence patient's outcome. Other factors rather than the level of resistance may have been important in the definition of this outcome, and the presence of polymorphisms and unknown mutations may help modulate the drug-resistance level induced by resistance mutations.

Resumo

A infecção pelo citomegalovírus humano (HCMV) é reconhecida como a principal causa de morbilidade e mortalidade em doentes que recebem transplante de células estaminais hematopoéticas (HSCT). Atualmente, existem quatro fármacos antivirais (Ganciclovir, Valganciclovir, Foscarnet e Cidofovir) para prevenir e tratar a infecção e doenças causadas pelo HCMV. Contudo, uma terapia antiviral prolongada pode levar à resistência antiviral associada ao desenvolvimento de mutações nos genes virais fosfocinase (*UL97*) e/ou DNA polimerase (*UL54*). A caracterização das mutações de resistência aos fármacos anti-HCMV é importante no sentido de melhorar o tratamento dos doentes. Este estudo teve como objectivo caracterizar as mutações nos genes *UL97* e *UL54* em vinte e dois doentes com infecção HCMV, que foram submetidos a HSCT alogénico, no Instituto Portugês de Oncologia do Porto. Os genes *UL97* e *UL54* foram amplificados por *nested* PCR e sequenciados para caracterização genética. A informação genética foi comparada com estirpes HCMV de referência e descrita como mutações de resistência, polimorfismos ou mutações de fenótipo desconhecido. Foram identificadas mutações de resistência em sete doentes (32%): cinco (23%) continham mutações no *UL97* (C592G, A594V, L595W e C603W) e dois (9%) continham mutações no *UL54* (P522S e L957F). As mutações de resistência encontradas no gene *UL97* estão entre as mutações mais frequentes nas estirpes resistentes. As mutações do *UL54* foram detectadas na ausência de mutações no *UL97* e numa frequência menor. A mutação P522S é uma das mais frequentes no *UL54* e a mutação L957F apenas tem sido reportada em estirpes laboratoriais. Vários polimorfismos foram encontados em ambos os genes, em combinação com as mutações de resistência. Adicionalmente, também foram identificadas mutações de fenótipo desconhecido, as quais poderão ser importantes no aparecimento da resistência antiviral. As mutações de resistência, que conferem diferentes níveis de resistência, levaram a uma resposta inadequada à terapia com impacto no *outcome* dos pacientes. Outros factores podem também ter sido importantes para o *outcome* dos doentes, sendo que a presença de polimorfismos e mutações de fenótipo desconhecido poderão ter ajudado a modular o nível de resistência das mutações.

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Abbreviations and Acronyms List

A	
aa	Amino acid
ACV	Acyclovir
Ag	Antigenemia
aGVHD	Acute GVHD
allo-HSCT	Allogeneic HSCT
AIDS	Acquired immune deficiency syndrome
ATG	Anti-thymocyte globulin
ATP	Adenosine triphosphate
auto-HSCT	Autologous HSCT
B	
BMT	Bone Marrow Transplant
bp	Basepairs
C	
CDV	Cidofovir
cGVHD	Chronic GVHD
CNS	Central nervous systems
CsA	Cyclosporine
D	
D	Donor
DB	Dense bodies
DNA	Deoxyribonucleic Acid
DOI	Duration of infection
dsDNA	Double stranded DNA
E	
E	Early
EBV	Epstein Barr Virus
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
F	
FDA	Food and Drug Administration
FOS	Foscarnet

G

gB	Glycoprotein B
gC	Glycoprotein Complexes
GCV	Ganciclovir
gH	Glycoprotein H
GI	Gastrointestinal
gL	Glycoprotein L
gM	Glycoprotein M
gN	Glycoprotein N
gO	Glycoprotein O
gp	Glycoprotein
GVHD	Graft-versus-host disease

H

HAART	Highly active antiretroviral therapy
HCMV	Human Cytomegalovirus
HHV-5	Human Herpesvirus 5
HHV6	Human Herpesvirus 6
HHV7	Human Herpesvirus 7
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HSCT	Hematopoietic stem cell transplant
HSV-1	Herpes simplex virus type 1

I

IC50	Half Maximal Inhibitory Concentration
IE	Immediate early
IgG	Immunoglobulin G
IPO Porto	Portuguese Institute of Oncology of Porto
IRL	Inverted repeat long
IRS	Inverted repeat short
IV	Intravenous
IVIGs	Intravenous Immune Globulin

K

kb	Kilobase
kDa	Kilodalton

L

L	Last

M

MIEA	Major Immediate Early Antigen
MRA	Mutation Resistance Analyzer
mRNA	Messenger ribonucleic acid

N

NA	Nucleic Acid
NIEPS	Noninfectious enveloped particles

O

ORFs	Open reading frames

P

PCR	Polymerase chain reaction
pH	Potential of hydrogen
PMNL	Polimorphonuclear leukocytes

R

R	Recipient
Rb	Retinoblastoma
RT-qPCR	Real time quantitative polymerase chain reaction

S

SOT	Solid organ transplant

T

TRL	Terminal repeat long
TRM	Transplant-related mortalitiy
TRS	Terminal repeat Short
TTI	Time to infection

U

UL	Unique long
US	Unique short
UV	Ultraviolet

V

VGCV	Valganciclovir

W

WBC	White Blood Cell

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Chapter 1

Literature's Review

1.1. Human Cytomegalovirus (HCMV)

In 1881, Ribbert described the presence of large intranuclear inclusions in kidney sections of a stillborn infant with congenital syphilis, who thought at that time that were caused by protozoal infections [1, 2]. These typical morphological alterations, known as "cytomegalic inclusions", were also reported by several authors during the early 1900s, and were attributed to either syphilitic or protozoan infection [3]. Nevertheless, it was only in 1921 that Goodpasture and Talbert have suggested that these "cytomegalic inclusions" could be caused by a viral agent [1, 4, 5]. In fact they suggested that the occurrence of these morphologic alterations in the liver and kidney without the presence of inflammatory signs appeared to indicate that the causal agent could be transported by the bloodstream [1, 4, 5]. Furthermore, in 1950, Smith and Vellios also showed that this infection could be spread in the utero [4].

Despite the evidences for a viral association, it was only in 1956 that Smith *et al*. and Rowe *et al*. followed by Weller *et al.*, in 1957, were able to isolate human CMV strains from the human salivary gland [4]. Later in 1960, Weller *et al.* proposed the term "cytomegalovirus" [1, 4]. Since then, human cytomegalovirus (HCMV) has been recognized as one of the most common opportunistic pathogens, especially found in immunocompromised patients as *human immunodeficiency virus* (HIV)-infected or transplanted patients [2, 4].

1.1.1. General characteristics

HCMV is a ubiquitous virus that belongs to the *Herpesviridae* family [6, 7] sharing with other herpesviruses a similar virion structure (icosahedral nucleocapsid, surrounding

tegument layer, and an envelope containing embedded viral glycoprotein complexes), a double-stranded DNA genome, kinetics of viral gene expression, persistence for the lifetime of the host after primary infection, reactivation from latency, and reinfection [8-14]. Within *Herpesviridae* family, HCMV belongs to the *betaherpesvirinae* subfamily and is alternatively known as Human Herpesvirus 5 (HHV-5) [12]. It is considered the betaherpesvirus prototype due to the tropism for salivary glands, the restricted host range and the slow replication that leads to slow spread of infectious particles in cell culture [8, 15, 16]. Even though the immune system keeps HCMV infections under control, a total HCMV clearance is rarely achieved, and the viral genome remains in a latent state [13, 17]. In fact, members of *betaherpesvirinae* subfamily are characterized by latent infections which are maintained in myeloid lineage cells [6]. Especially in cases of immune suppression, the reactivation of HCMV from latency is a key step in the pathogenesis of HCMV infection [6, 10-12, 17].

1.1.2. Structural characteristics

The mature HCMV virion particle has between 150 and 200 nm of diameter and is structurally more complex when compared to other members of the *Herpesviridae* family [1, 7, 18]. Structurally, a mature virion consists of a double-stranded linear DNA genome within a 100 nm diameter complex icosahedral nucleocapsid, surrounded by a lipid envelope containing embedded viral glycoprotein complexes [6, 18, 19]. Between the nucleocapsid and the envelope there is the tegument, an amorphous structure with some degree of organization which contains viral phosphoproteins – Figure 1 [6, 18, 20].

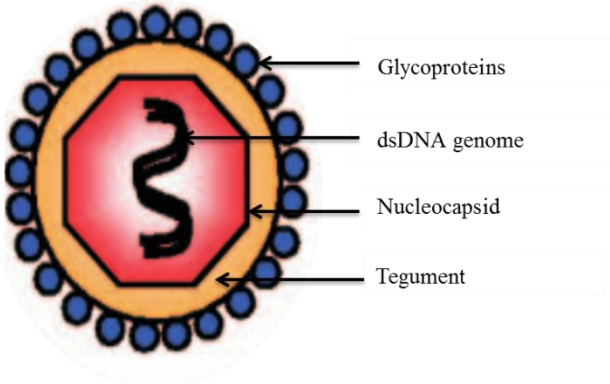


Figure 1: Structure of a mature HCMV virion**.** dsDNA – double stranded DNA is packaged in an nucleocapsid, surrounded by the tegument layer, and enclosed in the host-derived lipid envelope studded with virally encoded glycoproteins. Adapted from [6].

HCMV-infected cell cultures are characterized by the production of infectious virions and two other types of particles: noninfectious enveloped particles (NIEPS) that are very similar to infectious virions but lack viral genomes packaged within the icosahedral nucleocapsid; and dense bodies (DB) that are composed by several tegument proteins, but lack an assembled nucleocapsid and viral genome. Depending upon the viral strain and the number of infectious cycles in culture, the ratios of the three forms of HCMV particles released from infected cells vary [8, 18, 20].

The virion structural proteins, mainly proteins of the capsid, have been described as having homology to other herpes virus [7]. The capsid is an icosahedral structure consisting of 12 pentons, 150 hexons, and 320 triplexes of proteins that self-assemble into an icosahedral structure [7]. Of the more than thirty viral proteins found in the complete infectious virion, at least four proteins constitute the capsid: major capsid protein (pUL86); minor capsid protein (pUL85); the minor capsid protein-binding protein (pUL46); and the small capsid protein (pUL48-49) [7, 18, 21]. The pUL86 is the most abundant protein component of the capsid (960 copies) and forms the penton and hexons of the icosahedral capsid. The pUL85 (two copies) and pUL46 (one copy), form the triplexes that are located between the pentons and hexons. The smallest capsid protein (pUL48-49) has been shown to decorate the hexons of the capsid and is essential for the assembly of infectious virions, perhaps through interactions with tegument proteins [7, 22].

Among herpesviruses, HCMV has the largest coding capacity for glycoproteins. At least 57 potential glycoproteins are encoded by AD169 laboratory strain**,** while clinical isolates and Toledo laboratory strain, contain an additional 13 open reading frames (ORFs) that may also encode glycoproteins [23-25]. While some glycoproteins are proposed to play specialized functional roles tailored to replication and pathogenic features in the biology of HCMV infection, others are likely functionally redundant [24, 25]. The phospholipid envelope contains 6 genes encoded glycoproteins: gpUL55 (gB), gpUL73 (gN), gpUL74 (gO), gpUL75 (gH), UL100 (gM), and gpUL115 (gL). These glycoproteins play essential roles in virus entry into host cells, cell-to-cell interaction and virion maturation [25-27]. Studies have revealed that the gB, gN, gM, gL and gH glycoproteins have an essential role for the production of infectious virus, in which an disruption of ORFs results in the failure to produce infectious progeny [7, 21]. Only gO is dispensable for viral growth in culture fibrobasts [21]. The glycoproteins of the six major glycoprotein-

encoding genes, associate to form three glycoprotein complexes: gCI, composed of homodimeric gB molecules linked by disulfide bonds; gCII, comprises gM, a type III membrane protein, and gN, a type I membrane protein; and gCIII, composed of gH heterotrimer plus gO and gL [7, 23-25, 28]. These glycoprotein complexes are highly conserved within herpesviruses and function as membrane glycoproteins. At least two glycoprotein complexes (gCI and gCIII) are required for viral entry into cells [27]. While gCI appears to be the primary glycoprotein involved in attachment to the cell, gCIII is implicated in HCMV and host cell membrane fusion [21]. Very little is known about the role of components of the gCII complex, which have been identified as heparin binding proteins of the envelope [23].

The remaining 20-25 structural proteins are enclosed in the tegument layer of HCMV virions, which is characterized by an amorphous layer between the nucleocapsid and the envelope [18, 20]. The tegument appears to be formed by the sequential addition of proteins collected from the nucleus and the cytoplasm of infected cells. Some are expressed only in the nucleus (ppUL69), others only in the cytoplasm (ppUL32/pp150 or ppUL99/pp28), and others (ppUL53 and ppUL83/pp65) are expressed in the nucleus but are often found in the cytoplasm [29].

Most tegument proteins are phosphorylated and are highly immunogenic [18, 20]. There are five predominant proteins in the tegument: the basic phosphoprotein ppUL32, the high molecular weight protein ppUL48, the high molecular weight-binding protein ppUL47, the upper matrix protein ppUL82 and the lower matrix protein ppUL83 [30], while more than 20 proteins have been identified at lower concentrations, including gene products of *UL99*, *UL97*, *UL26*, *UL35* and *UL88* [31]. Due to its large amounts, pp65 is the target antigen in antigenemia assays for rapid diagnosis of HCMV clinical infections [21]. The tegument proteins are thought to be involved in the maturation of virions, influence viral and cellular events in the early stages of infection, such as release of viral DNA from disassembling virus particles or the regulation of viral and cellular gene promoters [7, 18, 20, 21]. The tegument ppUL99/pp28 protein appears to be essential for virus replication by providing an essential function for virion envelopment, while pUL94 contributes for the secondary envelopment of virions [32, 33]. Furthermore, ppUL69 and ppUL82/pp71 are transactivators of both viral and cellular gene expression contributing for the dysregulation of cycle progression [7, 20]. pp71 has been shown to transactivate immediate early viral promoters as well as target cellular retinoblastoma (Rb) family

members for degradation and ppUL69 has been shown to restrict cell cycle progression [21, 34, 35].

1.1.3. Genetic characteristics

The HCMV genome is the largest of all herpesviruses with approximately 230 000 basepairs (bp) [1, 19, 36, 37]. The organization of the viral genome consists of two covalently linked segments, (UL as unique long and US as unique short) bounded by repeat sequences located internally (internal repeat sequences binding the long and short segments, IRL and IRS, respectively) and terminally (terminal repeat bounding the long and short segments, TRL and TRS, respectively) - Figure 2 [11, 19, 37-39].

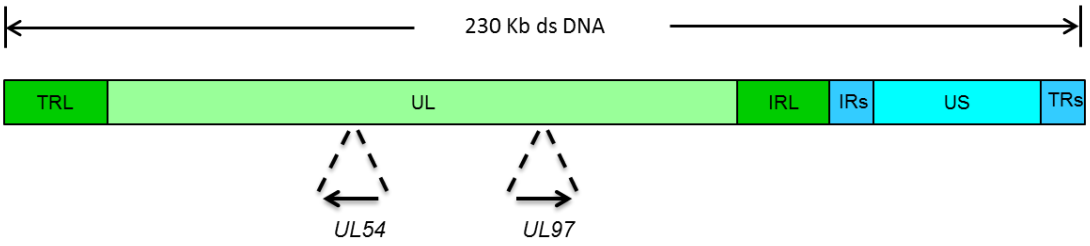


Figure 2: Structural components of the HCMV genome. UL and US indicate unique long and unique short regions, respectively. Repeat sequences are denoted by: TR - terminal repeat, IR – internal repeat. Positions of genes of interest are indicated by dotted lines. Arrows show the direction of the open reading frame (ORF) for each gene. Adapted from [19].

Like the genome of other herpes virus, both segments can undergo inversion, resulting in four isomers of the viral genome [1, 7, 21, 39, 40]. Inversion of UL and US regions is mediated by direct repeat elements (a, b, c) at the genome termini and by inverted repeat elements at the UL-US junction (a´, b´, c´) - Figure 3 [21, 39].

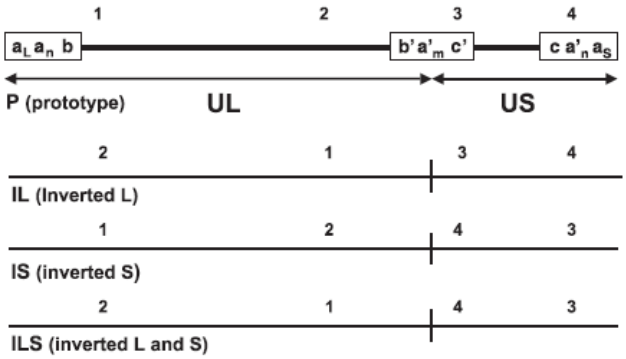


Figure 3: Structure of the four HCMV genome isomers, which are produced by inversion of UL and US regions **[21].**

The HCMV genome contains a single origin of replication, and like all human herpesviruses encodes a complete package of genes required for viral DNA replication including the DNA polymerase gene (*UL54*) [19, 36].

Viral genes conserved between different betaherpesviruses are most frequently found in the UL region of the genome, whereas genes located within the US region in general, encode functions that are specific for betaherpesviruses, including the host restriction of HCMV [7]. A significant number of genes in the UL region encode proteins that likely play key roles in the tissue and cellular distribution of the virus *in vivo*, yet are dispensable for *in vitro* replication [7].

The number of virus genes encoded by the genome of HCMV is greater than in other herpesviruses, but it remains unclear how many ORFs are expressed [40]. In fact, depending on the virus strain and the used prediction method, the HCMV genome has been estimated to encode between 192 and 252 ORFs [41]. Studies have assigned functional roles to some HCMV ORFs, nevertheless, the products of more than 50 HCMV ORFs have been considered dispensable for productive replication *in vitro* [21, 42]. Approximately 25% of the herpesvirus-conserved ORFs appear to encode functions related to viral DNA metabolism and replication, whereas the remaining 75% are thought to be involved in the maturation and structural organization of virions [21].

The AD169 laboratory strain was the first and the only completely sequenced HCMV strain, nevertheless it seems to have shorter genome than do many clinical isolates [36]. Analysis of its genome has revealed that it encodes 225 ORFs of about 100 or more amino acids (aa) residues in length, named according to the region and the numerical order in which they occur [19, 42]. There have been described the existence of unique ORFs, UL1-154 (with some ORFs receiving fractional designations such as UL21.5) and US1-36; and also repeated ORFs, J1L/J1I/J1S, TRL1-14/IRL1-14; and IRS1 plus TRS1 - Figure 4 [42]. Additional ORFs have been identified in two laboratory strains (*Towne* and *Toledo*) and in clinical isolates [42]. The *Toledo* strain, as well as some clinical isolates contains an additional 15 kilobases (kb) of DNA that is absent in the genomes of AD169 and *Towne* strains [19, 36, 41]. This large block of DNA seems to contain 19 genes encoding for viral glycoproteins and other specialized functions [36, 41].

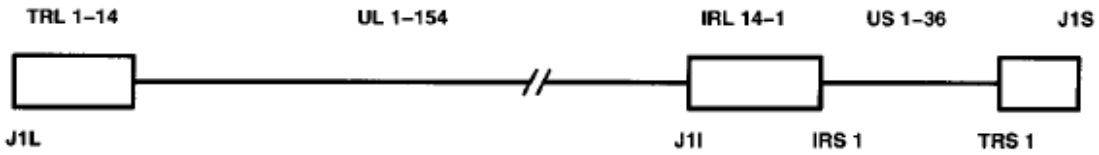


Figure 4: Schematic diagram of the linear, double-stranded DNA of HCMV. Repeated genes (J1L/J1I/J1S, TRL1-14/IRL1-14) and partially repeated genes (IRS1 and TRS1) are represented by rectangles, whereas unique gene blocks (UL1-UL154 and US1-US36) are designated by a line [42].

1.2. HCMV Infection

1.2.1. Epidemiology

HCMV is a common virus with unknown seasonal predominance and its epidemiology varies in different regions of the world and between socioeconomic and age groups [1, 43, 44]. Generally, the prevalence of HCMV infection is higher in developing countries and among persons of lower socioeconomic status in developed nations [1, 45]. HCMV infection is considered important in certain risk groups such as immunocompromised individuals and pregnant women [43, 45].

The incidence of HCMV infection in the general population ranges from 36 to 90%, but the overall seroprevalence in developed countries is estimated to be in the range of 30–70% [44, 46]. HCMV infection gradually increases with age, showing the lifelong risk of acquiring HCMV infection [1, 43, 45]. The prevalence of HCMV seropositivity in younger age varies according the social standing, being higher in lower economic classes [41, 47]. Racial differences have also been described, with higher seroprevalence found in African Americans and Hispanics than in Caucasians [1, 45]. An increased seroprevalence of HCMV has also been described in women attending sexually transmitted disease clinics and in young homosexual males [1, 44].

1.2.2. Source of Transmission

HCMV is easily transmitted orally, through sexual intercourse, breastfeeding, placental transfer, blood transfusion or transplantation [44, 46]. The unsuspecting host is thus able to spread the virus both vertically and horizontally [1, 21, 48], since it is excreted

through body fluids (urine, saliva, tears, semen, milk, and cervical secretions) for months to years. [12, 43, 48].

Vertical transmission of HCMV can occur in three different ways: transplacental, intrapartum or breastfeeding [1]. Transplacental transmission can occur both in women infected for the first time during pregnancy and those infected long before conception [21]. A primary infection in the first 16 weeks of pregnancy is associated with higher rate of damage in fetal development [43]. Infection during delivery is due to shedding from the vagina or cervix, followed by ingestion of infected secretions by the offspring [21]. Breastfeeding is considered the most common mode of transmission to children and plays an important role in the epidemiology of HCMV infection as the virus is reactivated during lactation in nearly every seropositive mother [1, 43, 48].

Epidemiologic studies support the classification of HCMV as a sexually transmitted infection, consistent with excretion of this virus in cervical secretions, vaginal fluid, and semen [1]. Furthermore, HCMV has also been described as capable of being transmitted through blood or transplants [1]. The association between the acquisition of HCMV primary infection and blood transfusion was first suggested in 1960 as it is assumed that the virus is latent in the blood cells of healthy donors and is reactivated following transfusion when they encounter an allogeneic stimulus [1, 21].

1.2.3. Infection and replication Cycle

HCMV infection starts with the attachment of HCMV membrane glycoproteins to the host cell surface receptors by a pH-independent mechanism [1, 3, 20, 21, 37] followed by the uncoated of viral capsids and rapid translocation of viral DNA into the nucleus – Figure 5 [3, 21].

The mechanism by which HCMV entry proceeds is cell type-dependent and occurs via fusion with the plasma membrane (on fibroblasts) or by acid-mediated endocytosis (in epithelial and endothelial cells). Both pathways require the viral gH/gL complex, however further characterization indicates that while gCIII (gH/gO/gL) is involved in binding of HCMV to fibroblasts, a complex consisting of gH/gL/UL128-131 plays a significant role in attachment and entry into epithelial and endothelial cells [1, 7, 20, 49].

Virus attachment and penetration are fast and efficient in both permissive and nonpermissime cell types [3, 21, 49]. The viral glycoprotein B (gB), encoded by *UL55*

gene, is the primary viral ligand that interacts with two separate binding sites: the heparin sulfate proteoglycans and a non-heparin receptor [21]. During the initial virus-cell interactions, HCMV attaches to the cell surface by low-binding of gB to heparan sulfate proteoglycans. The subsequent interaction of gB with its non-heparin receptor increases the stability of HCMV bind to cell surface [21]. However, the fusion of the viral envelope with the cell membrane to allow viral penetration is thought to require a further event mediated by the heteroligomeric gCIII (gH/gL/gO) complex with unidentified receptors - Figure 5 [7, 27].

After translocation into the nucleus, viral mRNA is transcribed and the transcripts exported from the nucleus to be translated by cellular machinery [3, 50]. Nevertheless, while some translated viral gene products are solely cytoplasmic proteins, others may return to the nucleus to regulate viral replication and cellular control [3, 50]. DNA replication, capsid assembly and DNA packaging occurs in the nucleus and viral assembly, which includes tegumentation and envelopment of new viral capsids, occur in the cytoplasm- Figure 5 [3, 51].

Similar to other herpesviruses, HCMV DNA replication begins 16 to 24 hours after infection [37] involving temporally ordered viral gene expression [12, 19, 37, 38]. The first transcribed viral genes are the immediate early (IE or α) genes, which are mainly transcriptional regulators, and have the ability to be transcribed in the absence of *de novo* protein synthesis, and are assumed to carry out key regulatory functions in permissive as well as in latent infection [1, 3, 19, 37, 38]. Expression of these genes is required for the transcription of early genes (E or β) [3, 12, 19, 37, 38]. Early genes are divided into two subclasses (β1/E and β2/E-L) and encode multiple proteins required for synthesis, processing and repair of DNA, capsid assembly, encapsidation, and establishment of immune evasion in the productively infected cell [1, 8, 12, 19, 37, 38]. HCMV replication occurs after circularization of DNA and DNA synthesis starts by rolling circle replication which can undergo genomic inversion [47]. HCMV genome, unlike other herpesviruses, does not encode deoxyribonucleotide (dNTPs) biosynthesis enzymes, thus it has developed strategies to stimulate the biochemical pathways involved in the biosynthesis of DNA precursors [21]. This feature is considered crucial for its productive replication in the quiescent or terminally differentiated non-dividing cells [21]. Several *trans* acting factors are required for origin (*ori*Lyt)-dependent DNA replication which include the six

herpesvirus-conserved ORFs that provide the core replication proteins for viral DNA replication [8]. Among them, the single-stranded DNA-binding protein ppUL57 prevents the reannealing of DNA strands, followed by the unwinding by the helicase-primase complex, composed by primase (pUL70), helicase (pUL102) and primase-associated protein (pUL105) encoded by *UL70*, *UL102*, and *UL105*, respectively. The DNA polymerase processivity factor pUL44, encoded by UL44, prevents the dissociation of DNA polymerase pUL54, which is encoded by *UL54*, from the template [8, 19, 38]. The amino acid sequences of all of these proteins are highly conserved among HCMV strains and pUL54 3'-5'exonuclease activity (proofreading) is responsible for the high fidelity of the replication, which results in a low mutation rate [19]. The Late (L or γ) proteins are the last class of gene products expressed during HCMV replication and can be divided into two classes: gamma 1/leaky-late (γ1) and gamma 2/true-late (γ2) [8, 38]. Proteins encoded by these genes have mainly structural roles and contribute to the assembly and morphogenesis of the virion [1, 3, 12, 19, 37, 38]. Their transcription begins more than 24 hours after infection and requires prior viral DNA replication [37]. The late genes *UL94*, *UL99*, and also *UL32* tegument proteins are essential for late events in virion assembly in the cytoplasm [32, 52].

The growth cycle of HCMV is relatively slow and viruses are not released until 72 to 96 hours [37]. Despite the fact that the precise molecular events of capsid assembly and DNA packaging are not yet understood, these processes are thought to occur in the nucleus – Figure 5 [53]. Three distinct capsid types, termed A, B, and C, are found in HCMV-infected cells [53]. The packaging of the DNA genome leads to the egress of the capsid scaffold and gives rise to a mature capsid - Figure 5 [21, 51]. Errors in packaging can result in cytoplasmic accumulation of non-infectious enveloped viral particles containing capsids lacking scaffold or DNA and capsids with only scaffold protein cores [54]. In fact, experiments revealed an important role for *UL97* kinase (pUL97) in capsid assembly [19, 55]. Optimal nuclear egress requires active pUL97 and its detection correlates with abnormal subcellular distribution of viral structural protein assembly sites, both in the nucleus and in perinuclear structures, and consequent reduction in viral yield [19, 55]. Capsids are initially assembly through budding at the nuclear membrane, where they acquire a primary envelope derived from its inner leaflet. Then, they cross the lumen, fuse with the outer leaflet of the nuclear membrane or the endoplasmic reticulum (ER) membrane, lose their primary envelope, and move into the cytoplasm, where HCMV virion

particles acquire their tegument - Figure 5 [21]. Final envelopment of tegument-coated HCMV capsids occurs into a Golgi-derived secretory vacuole specifically destined for the plasma membrane and not marked for degradation in lysosomes or endosomes [14, 21]. Finally, the egress of infectious viral particles occurs by the fusion of secretory vacuoles containing HCMV viral particles with the cell membrane after transport via the vesicle trafficking pathways - Figure 5 [14, 21].

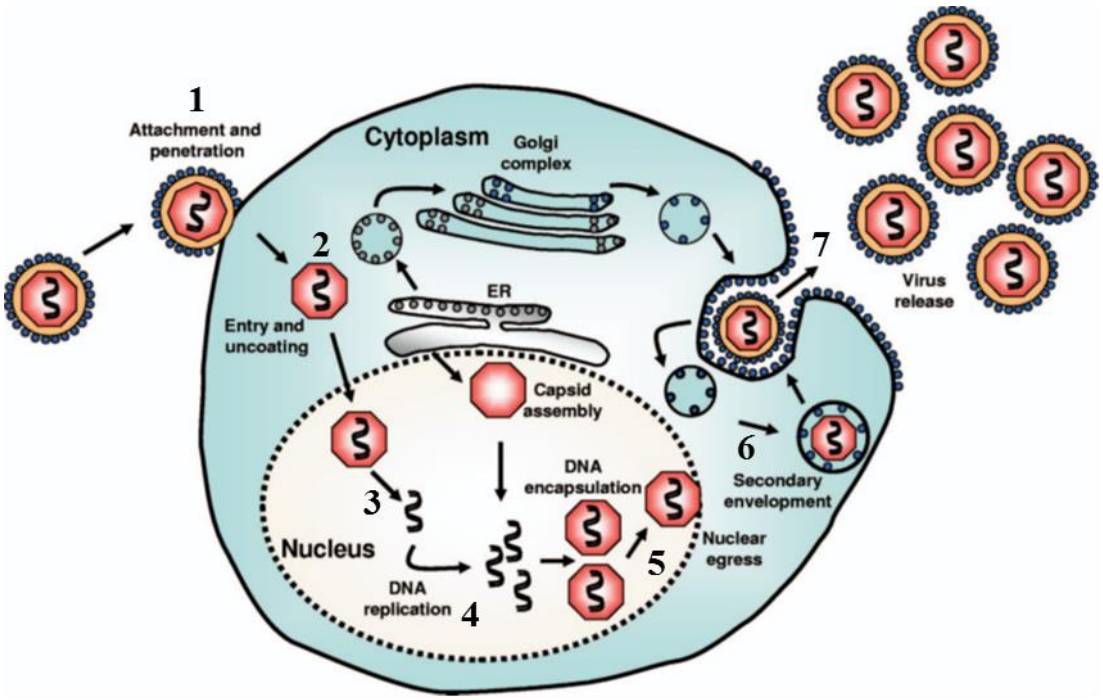


Figure 5: HCMV Life Cycle. 1) HCMV attachment to the cell membrane with penetration via endocytosis or fusion at the plasma membrane; 2) Virion contents are released into the cytoplasm; 3) nucleocapsids are translocated into the nucleus, where viral DNA is released; 4) Viral replication and maturation follow the stimulation and parallel accumulation of viral synthesis function; 5) the encapsulation of replicated viral DNA as capsids, which are then transported from the nucleus to the cytoplasm; 6) envelopment of tegument-coated HCMV capsids occurs into a Golgi-derived secretory vacuole in the cytoplasm; 7) egress process that leads to virion release by exocytosis at the plasma membrane. Adapted from [6].

1.2.4. HCMV Infection and Disease

HCMV infection is in the majority of cases asymptomatic, since the virus is maintained in a state of latency or low level shedding that is clinically undetectable [13, 19, 46]. Symptomatic infections in immunocompetent individuals are rare [4]. However, the groups at higher risk of HCMV infection and associated disease development are individuals with compromised or immature immune systems, including those infected with HIV, transplant recipients and congenitally-infected infants. In these patient groups,

HCMV infection can lead to HCMV-associated disease resulting in significant morbidity and mortality [8, 16].

Infants infected in utero are at risk for several congenital abnormalities and sensorineural hearing loss [13, 36, 40, 45, 46]. In patients who have *acquired immune deficiency syndrome* (AIDS) most commonly developed HCMV retinitis that leads to vision loss [13, 36, 46]. In transplant recipients, HCMV has both direct effects, resulting from viral invasion of organ systems, and indirect effects on the immune systems [46, 56]. The direct effects of HCMV primary infection or reactivation in organs are the development of end-organ diseases such as pneumonia, hepatitis, pancreatitis, gastrointestinal disease, retinitis, encephalitis, colitis, esophageal ulcers and others [13, 21, 36, 56]. Regarding the indirect effects, they are often associated with increased risk of other infections, bacterial and fungal, and promote acute graft rejection [46]. In fact, HCMV has repeatedly been associated with rejection after solid-organ transplantation and with graft-*versus*-host disease (GVHD) after hematopoietic stem cell transplant (HSCT) [19, 36, 56].

1.3. HCMV and HSCT

1.3.1. Epidemiology and Characteristics

HCMV infection is the leading viral cause of morbidity and mortality in patients who receive hematopoietic stem cell transplant (HSCT) or solid organ transplant (SOT) (including kidney, liver, heart, heart-lung) [36, 46].

Studies have described that HSCT recipients have a higher prevalence of HCMV infection and associated diseases than SOT recipients [16, 57]. The incidence of HCMV infection following allogeneic HSCT (allo-HSCT) ranges from 32% to 70%, varying with the serological status of the recipient (R) and donor (D) [21]. Its incidence in seronegative recipients with seropositive donors (D+/R-) is lower than in seropositive recipients with seropositive donors (D+/R+), suggesting a transfer of adoptive immunity from donor to recipient. Thus, the most critical event is reactivation of a latent virus in seropositive recipients [15, 21, 46, 58-60]. In seronegative recipients, transmission mostly occurs through larger quantities of blood products that they receive [21].

A recent study from Portugal showed that 60.3% of allogeneic HSCT patients developed HCMV infection, mainly viral reactivations rather than primary infections (96.2% vs 3.8%, respectively) [61]. Typically, HCMV infection/reactivation appears within the first 100 days after transplant, both in allogeneic and autologous recipients, and affects mainly the lungs and the gastrointestinal tract [60, 61]. In HSCT recipients, late-onset HCMV disease (disease occurring >100 days after transplant) is similar to that observed in SOT patients, but with higher mortality [60, 62, 63]. Nevertheless, the increase in late-onset HCMV infections may be due to effective antiviral prophylaxis or preemptive treatment during the first 100 days after transplantation [58, 63, 64], by inhibiting the development of HCMV-specific T-cell lymphocyte response [62, 64, 65]. In fact, while prophylaxis and pre-emptive therapy are effective for the prevention of the HCMV disease during the antiviral treatment, cessation of therapy can result in the emergence of late-onset HCMV disease [58, 63].

1.3.2. Risk Factors

The risk of developing HCMV reactivation or disease in HSCT patients is associated with the type of transplant and its associated-complications [59, 61, 64, 65]. Indeed, not all individuals are at the same risk as it varies with age, underlying disease, source of stem cells, donor/recipient (D/R) HCMV serological status, type of immunosuppression, and occurrence of graft-*versus*-host disease (GVDH) [36, 58, 59, 61, 64-67]. Moreover, the risk factors for HCMV infection vary during the transplantation period and while some can be predicted prior the transplant, other are dependent on the outcome of the transplant - Figure 6 [58-60, 64, 65].

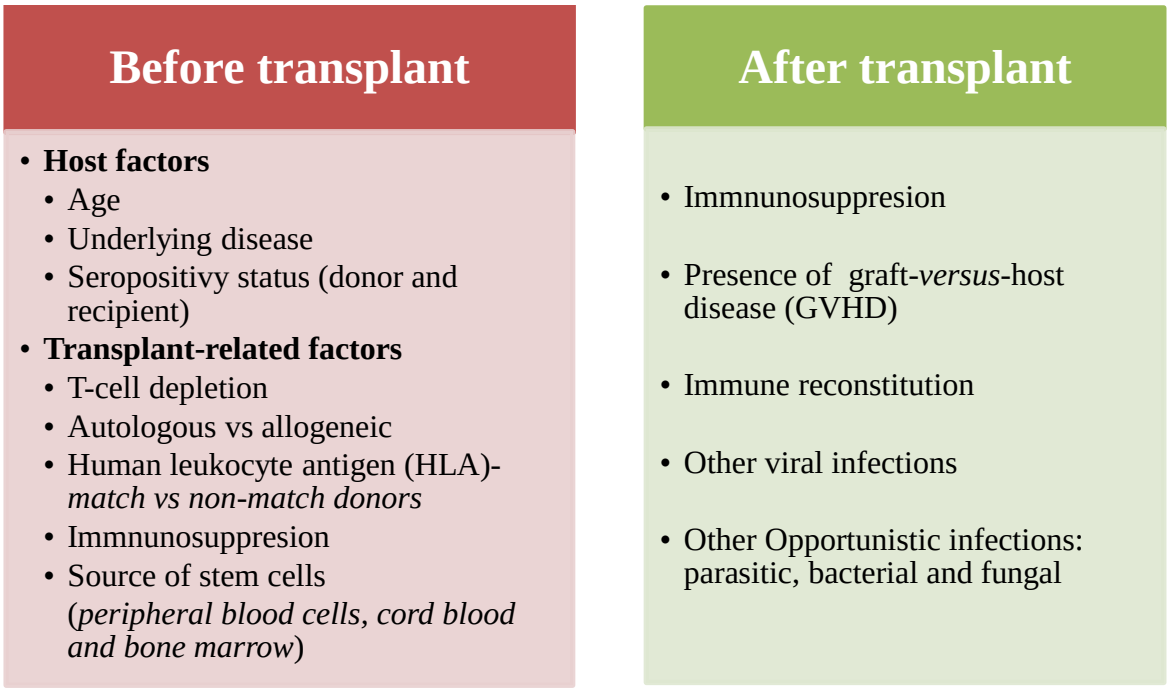


Figure 6: Conditions associated with the risk of developing HCMV infection, reactivation, or disease in each of the following phase of therapy in HSCT patients. Adapted from [65].

1.3.2.1. Before Transplantation

The risk factors that can be used prior transplantation to predict the occurrence of a HCMV reactivation/disease may be divided in host factors and transplant-related factors - Figure 6. Considering the host factors, it has been demonstrated that older ages represents a risk factor for developing HCMV reactivation/disease and for transplant-related mortality (TRM) [59, 61, 65]. The underlying disease and its treatment has not been specifically studied as a risk factor for development of HCMV reactivation/disease after HSCT, nevertheless, it has been demonstrated that the disease stage at the time of transplant is a highly significant predictor of mortality and that a diagnosis of chronic myelogenous leukemia in patients receiving T cells depleted from a HCMV–positive donor could represent a negative prognostic factor [59, 61, 65]. Furthermore, HCMV serostatus is considered mandatory in all transplant recipients and donors to evaluate the risk of HCMV reactivation/disease [58, 61, 66, 68, 69]. In autologous HSCT (auto-HSCT) recipients the probability of HCMV infection has been reported to be nearly 60% in seropositive patients and 23% in seronegative patients, however, the risk of developing HCMV reactivation/disease is lower than in allogeneic HSCT recipients [36, 65]. In allogeneic HSCT recipients, the risk of developing HCMV reactivation/disease has been reported as

5% of seronegative patients with seropositive donors (D+/R-), 14% of seropositive patients with seronegative donors (D-/R+), and 12% of seropositive patients with seropositive donors (D+/R+) [17, 59, 60, 65, 69]. Thus, the reactivation of HCMV occurs in nearly 80% of HCMV-seropositive recipients and 28% of seronegative recipients who receive a graft from a seropositive donor [65, 68]. Ljungman *et al.* suggests that seronegative patients that received grafts from seropositive donors have improved outcome [70], and in contrast, seropositive patients who receive grafts from seronegative donors have an increased risk of both repeated HCMV reactivation and disease [17, 59, 60, 65, 69]. Furthermore, seronegative recipients receiving transplant from seronegative donors have a very low risk of primary infection and a lower HCMV-related mortality [58, 59, 68].

Regarding the transplant-related factors, the type of transplant, Human leukocyte antigen (HLA) status, immunosuppression regimen, source of stem cells and T-cell depletion are considered important in the definition of risk for HCMV reactivation or disease - Figure 6 [17, 58, 60, 62, 64, 65, 67, 68]. Recipients of T cell-depleted allografts are the patients predominantly affected by HCMV reactivation, a rapid onset of HCMV-related symptoms, and a higher rate of fatal infections that may occur during the first 30 days after HSCT [58, 64, 66]. The risk of HCMV reactivation/disease is higher in recipients of transplants from an unrelated donor than in recipients of a related donor. However, in the allogeneic HSCT patients the risk of HCMV reactivation or disease is higher compared with patients receiving autologous HSCT [58, 60, 65, 66]. In recipients of unrelated or mismatched donor transplants, the increased risk of HCMV reactivation/disease is associated with a higher risk for HCMV-associated death and TRM, with higher mortality occurring in HCMV-positive patients receiving a transplant from negative donors [65]. Ljungman *et al*. also showed that allogeneic HSCT from seropositive donors was associated with better survival than from seronegative donors when the donors were unrelated, but not when the donors were HLA-identical siblings [70]. More aggressive chemotherapy regimens in transplant candidates have led to an increased infection of HCMV before transplantation [62]. Recipients of non-myeloablative stem cell transplants have a reduced risk of early HCMV infection and disease compared with standard myeloablative regimens [68]. However, they are also at an increased risk of late HCMV disease, mostly related to the use of antithymocyte globulin (ATG) or alemtuzumab (anti-CD52 monoclonal antibody) during conditioning chemotherapy [17,

60, 62]. A randomized study has showed, somewhat unexpectedly, that peripheral blood stem cell transplantation has not been associated with less HCMV infection and disease when compared to marrow transplantation [71]. However, in a non-randomized study, has been observed a moderate reduction in HCMV disease [72]. Cord blood transplantation is associated with similar rates of HCMV infection and has generated new populations of patients at high risk for HCMV reactivation and disease [60, 62]. However, blood products transfusion represents the main risk factor for HCMV acquisition in HCMV-negative patients receiving bone marrow from a HCMV-negative donor [65].

1.3.2.2. After Transplantation

Amongst the post-transplant related risk factors for HCMV reactivation or disease are the immune reconstitution, the development of GVDH, immunosuppression regime and the development of other infections - Figure 6 [17, 59, 60, 62, 64, 65, 68]. The development of HCMV disease occurs especially in HSCT recipients submitted to highly immunosuppressive regimens used to prevent rejection of the transplant, in particular those that lead to prolonged lymphocytopenia such as the use of fludarabine or analogues as well as alemtuzumab [60, 62]. In fact, the severity of the end-organ disease caused by HCMV is related to the degree of immune suppression [36, 60]. Furthermore, the immune reconstitution is also a significant factor, since while in SOT the host has the immune system working very early, in HSCT recipients the time to reach immune reconstitution may vary a lot [17, 64, 65]. Furthermore, allogeneic HSCT recipients, in contrast to autologous HSCT patients, are at a much higher risk of active HCMV infection because of the delayed recovery of T- and B-cell functions [17, 64]. In fact, studies have found HCMV-specific cytotoxic T-lymphocytes regeneration is dependent on HCMV serologic status, with both CD4+ and CD8+ T lymphocytes to be required for complete restoration of immunity [65]. However, the inability to control HCMV reactivation after allogeneic Bone Marrow Transplant (BMT) has been mainly associated with impaired function of antigen-specific CD8+ T cells rather than an inability to recover a sufficient numbers of HCMV-specific T cells [65]. In addition, it has been demonstrated that CD4+ T-helper cells regenerate relatively slowly following allogeneic BMT with subsequent limited help for CD8+ T cells to control HCMV replication and have observed lower HCMV-specific CD8 T-cell numbers during viral replication [17]. Therefore, HCMV pneumonia during the

first 120 days after HSCT is much more severe and life-threatening than it is in a patient after renal transplantation [36].

Another risk factor for HCMV reactivation or disease is the development of GVHD after transplantation and has been shown that acute and chronic GVHD significantly increases the risk of HCMV infection [17, 64, 65]. Moreover, patients that develop chronic GVHD (cGVHD), which is characterized by a severe combined cellular and humoral immunodeficiency, and acute GVHD (aGVHD), are at a prolonged risk to develop late-onset HCMV disease [60, 62, 64, 65].

Other viral infections, such as Human herpesvirus 6 (HHV6), Human herpesvirus 7 (HHV7) and Epstein Barr virus (EBV) have been implicated as risk factors for progression from active HCMV infection to HCMV disease and with reactivation of HCMV by suppressing the development of HCMV-specific immune responses [65]. In addition to viral infections, other opportunistic infections such as parasitic, bacterial, and fungal also emerge as common causes of HCMV reactivation/disease [58].

1.3.3. Clinical Features

HCMV end-organ disease is classified as of early onset (<100 days after transplant) or late onset (>100 days after transplant) [36, 60]. Pneumonia and gastrointestinal involvement are the most frequently described diseases caused by HCMV in HSCT recipients; nevertheless, there are other documented conditions such as hepatitis, retinitis, encephalitis, hemorrhagic cystitis, unexplained fever, endothelial damage, and thrombotic microangiopathy [17, 36, 55, 58, 60, 68].

The interstitial pneumonia caused by HCMV is the most common life-threatening infectious complication of allogeneic HSCT, defined by the presence of pulmonary disease, combined with HCMV found in bronchoalveolar lavage fluid or lung tissue samples. It is still a potentially fatal disease, which usually occurs within the first 120 days after transplantation, with decreasing incidence and severity after the initiation of routine antiviral prophylaxis or pre-emptive therapy after HSCT [17, 36, 65, 68]. The incidence of HCMV pneumonia ranges from 10% to 30% in allogeneic HSCT recipients and from 1% to 6% in autologous HSCT recipients. It has been described that 69% of cases occur early and 31% late after transplant [17]. HCMV pneumonia is associated with the following risk

factors: prolonged deficiency in HCMV-specific cytotoxic T-lymphocyte activity, recipient seropositivity, older age, presence of acute GVHD, use of cyclosporine (CsA) as GVHD prophylaxis, and the underlying disease [17, 36, 65]. Lymphocytopenia, male gender, and severe acute GVHD are also often referred as contributing for severe HCMV pneumonia [17, 36, 65].

Gastrointestinal (GI) HCMV disease has been described both in autologous and allogeneic HSCT as a combination of clinical symptoms in gastrointestinal tract, findings of macroscopic mucosal lesions at endoscopy, and the symptoms vary depending on the location of the disease [17, 36, 65]. It is characterized by an erosive and/or an ulcerative condition that can occur at any location in the GI tract, from mouth to rectum [36, 65]. The incidence rates for GI HCMV disease are of 2% at 2 years after HSCT, with higher frequency in allogeneic than in autologous HSCT recipients [17]. The risk factors for the development of GI HCMV disease include allogeneic transplantation, use of steroids, the presence of intestinal acute GVHD and the HCMV-seropositive recipients [17, 65].

1.4. HCMV infection management

1.4.1. Anti-HCMV Drugs

Currently, four antiviral drugs are used to prevent/treat HCMV infection and diseases. These antiviral drugs act by inhibiting effective HCMV DNA synthesis and have been shown to be effective in the prevention and/or treatment of HCMV infection and diseases: Ganciclovir (GCV), Valganciclovir (VGCV), Cidofovir (CDV) and Foscarnet (FOS) - Figure 7 [40, 46, 73-76]. Despite their clinical utility being limited by the efficacy, limited oral bioavailability, development of resistance in clinical practice, and associated toxicities, these drugs have been used to treat many forms of HCMV disease in immunocompromised patients [40, 46].

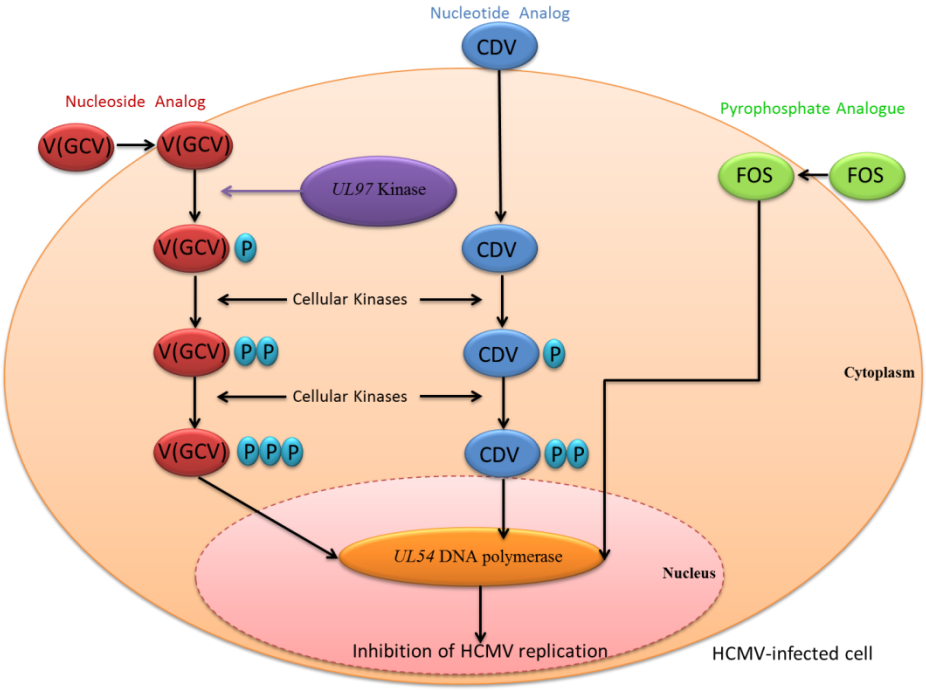


Figure 7: Action mechanisms of systemic antivirals approved for treatment of HCMV infection. GCV/VGCV requires phosphorylation by the phosphokinase (pUL97). After monophosphorylation by pUL97, the cellular kinases add two additional phosphates. GCV triphosphate is the active form of the drug incorporated into viral DNA by the viral DNA polymerase (pUL54). CDV is a monophosphate analog and does not require initial viral phosphorylation. Cellular kinases add additional phosphates to produce CDV diphosphate, which is incorporated into the viral DNA by pUL54 leading to termination of viral DNA replication. FOS is a pyrophosphate analog, which does not require activation and is not incorporated into the growing viral DNA chain. It blocks directly the release of pyrophosphate by pUL54 and therefore resulting in chain termination. Adapted from [19, 77].

1.4.1.1. Ganciclovir and Valganciclovir

Ganciclovir (GCV) or (9-[1, 3-dyhydroxy-2-propoxymethyl] guanine) was the first antiviral agent approved for the treatment of HCMV infection/disease, and remains the first-line treatment for HCMV infection/disease in immunocompromised patients [36, 46, 77, 78]. It is an inactive nucleoside analogue of guanosine and homologue of acyclovir (ACV) [10, 36, 40, 77]. GCV is converted to GCV triphosphate that is the active form of drug by a multistep process dependent on both viral and cellular enzymes - Figure 7 and Table I [19, 46, 78-80]. The two target viral proteins involved in GCV anabolism are: *UL97* and *UL54* [19, 40, 78]. The *UL97* gene of HCMV encodes a viral protein kinase that phosphorylates GCV to GCV monophosphate [10, 36, 40, 46, 77-79, 81]. Two subsequent phosphorylation steps are performed by host cellular kinases that result in the formation of the GCV triphosphate metabolite, which is a competitive inhibitor of the natural substrate (deoxyguanosine triphosphate) for the viral DNA polymerase encoded by *UL54* - Figure 7 and Table I [77, 78, 82]. GCV is not an absolute chain terminator, and short fragments of

HCMV DNA continue to be synthesized [36, 80], nevertheless its effects result from the ability to difficult and slow the elongation of viral DNA [36, 46, 81].

GCV has been shown to reduce the severity of HCMV retinitis, gastrointestinal disease and, to a lesser extent, pneumonia in SOT, HSCT and AIDS patients [21, 36, 40, 46]. GCV was initially approved by *Food and Drug Administration* (FDA) in 1989 for intravenous (IV) use [19, 46, 79]. Nevertheless, and despite its high bioavailability and therapeutic efficacy, IV use is limited since hospitalization is required for administration [46, 75]. Moreover, GCV use is limited by the occurrence of hematologic side effects, primarily neutropenia, and thrombocytopenia, mainly in the early phases of HSCT - Table I [17, 36, 46, 65, 68]. An oral capsule released in 1994 represented a major advance for treatment of HCMV retinitis, but could only be used as maintenance therapy, as the low bioavailability of the oral formulation was considered insufficient for induction therapy [19, 46, 79]. GCV poor oral bioavailability (5.6%) leads to the development of Valganciclovir (VGCV), a L-valyl ester prodrug which after oral administration is rapidly metabolized in the liver and intestinal wall - Table I [19, 36, 40, 46, 67, 77, 79]. The adverse effects of VGCV are similar to those of GCV, mainly, neutropenia and thrombocytopenia - Table I [36, 46, 67]. Nevertheless, VGCV has a much better bioavailability (60%) and is a suitable replacement for IV GCV in many clinical applications [19, 36, 40, 46, 67, 77, 79]. Thus, given the convenience to treat patients without hospitalization, VGCV tends to be widely used among transplant recipients, not only for prophylaxis, but also for preemptive and maintenance therapy [46, 67, 73].

Although GCV and VGCV have been effective in prevention and treatment of HCMV disease, the emergence of GCV/VGCV-resistant HCMV strains has posed a more significant threat due to an aggressive disease course and a greater mortality risk. Treatment options for these strains are limited, with FOS being recommended as the initial treatment option followed by CDV; nevertheless, these agents are known to have substantial side effects, the most notable of which is nephrotoxicity - Table I [83].

1.4.1.2. Foscarnet

Foscarnet (FOS) a pyrophosphate analogue, with the chemical name of phosphonoformic acid, which reversibly, and noncompetitively, inhibits the activity of the HCMV DNA polymerase - Table I [36, 40, 82]. FOS does not require intracellular

activation to exert its antiviral activity and is not incorporated into the growing viral DNA chain [19, 40, 77, 78, 80-82]. This noncompetitive inhibitor reversibly blocks the pyrophosphate binding site of the viral DNA polymerase and inhibits the cleavage of pyrophosphate from deoxynucleoside triphosphates - Figure 7 and Table I [19, 40, 46, 77-81]. FOS is administered as large volume intravenous solution since it must be present in high concentrations inside the cell to remain in contact with the viral DNA polymerase and inhibit DNA replication; hence, when the intracellular concentration decreases viral DNA synthesis resumes [10, 19, 36].

FOS was FDA approved in 1991 [10, 19] and despite its utility has been associated with nephrotoxicity and metabolic toxicity as well as renal failure, hypocalcemia, hypomagnesemia and hypophosphatemia - Table I [10, 36, 46]. Due to its side effects, FOS is considered a second-line therapy, preferred over GCV especially in patients with myelosupression or weak graft after HSCT, to treat patients with AIDS and HCMV retinitis who are failing GCV therapy due to viral resistance, or those who cannot be treated with GCV due to dose-limiting neutropenia or leucopenia [17, 36, 46, 68]. Furthermore, some studies refer that patients must be on long-term maintenance regimens with IV FOS to prevent the relapse or progression of HCMV disease [21, 36].

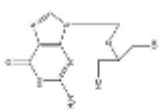
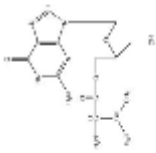
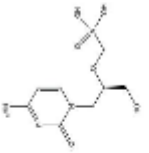
1.4.1.3. Cidofovir

Cidofovir (CDV) is a monophosphate nucleotide analogue, with the chemical name ([*S*]-1-[3-hydroxy-2-phosphonylmethoxypropyl] cytosine) [36, 40, 46, 78, 80]. Because a single phosphate-like group is already present in this monophosphate analog, CDV does not require initial phosphorylation by pUL97 kinase but is dependent on diphosphorylation by cellular kinases for activation - Figure 7 and Table I [19, 40, 80, 81]. Cellular kinases add the additional phosphate group to CDV, converting it into CDV diphosphate, an analog of deoxycytosine [46, 77, 81]. Similarly to GCV, the incorporation of CDV-diphosphate into viral DNA causes a slowing and subsequent cessation of HCMV DNA replication - Figure 7 and Table I [77, 78, 81]. Sequential incorporation of two CDV molecules completely inhibits further synthesis of HCMV DNA since it cannot be excised by pUL54 3-to-5 exonuclease activity [84].

CDV was FDA approved in 1996 as an IV formulation for the treatment of a broad-range of DNA viruses infections, including all the herpesviruses [40, 68]. CDV oral bioavailability is less than 5% [19, 46]; nevertheless it has a very long intracellular half-life when compared with GCV and FOS [36, 68]. Despite its efficacy as an anti-HCMV agent, due to the poor oral bioavailability and concerns about dose-related nephrotoxicity, lipid ester analogs, including as hexadecyloxypropyl-CDV (CMX001) and octadecyloxyethyl-CDV, are being tested against herpesviruses – Table I [46].

CDV has excellent activity against HCMV and has been reported to be effective in the treatment of HCMV retinitis in AIDS patients [40, 46] . CDV has also been studied for HCMV infection and disease in allogeneic HSCT [36], however, it has several side-effect such as nephrotoxicity and myelosuppression [17, 46, 68], and therefore it is considered a third-line agent for HCMV infection – Table I [17].

Table I: Antiviral agents used to prevent/ treat HCMV infection and disease. Adapted from [17].

Antiviral Drug	Class	Action Mechanisms	Adverse events	Resistance mutations
Ganciclovir (GCV) 	Nucleoside analogue	Acts via competitive inhibition of DNA synthesis	Fever, rash, diarrhea, hematologic effects	*UL97* protein Kinase; *UL54* DNA polymerase, mostly concomitant with UL97; *UL54* codons 375-540, cross-resistance with CDV; *UL54* codons 756-809, cross-resistance with FOS.
Valganciclovir (VGCV) 	L-valyl ester prodrug	Activated in the gut and liver to GCV, and has same mechanism of action		
Foscarnet (FOS)	Pyrophosphate analog	Reversibly blocks the pyrophosphate binding site of the viral DNA polymerase and inhibits the cleavage of pyrophosphate from deoxynucleoside triphosphates	Nephrotoxicity, renal failure, hypocalcemia, hypomagnesemia, and hypophosphatemia	*UL54* DNA polymerase; *UL54* codons 756-809, cross-resistance with GCV; *UL54* codons 812-987, cross-resistance with GCV and CDV.
Cidofovir (CDV) 	Nucleotide analogue	CDV is converted to the CDV diphosphate acts as a competitive inhibitor of the HCMV DNA polymerase, causing premature chain termination in viral DNA synthesis	Primarily severe nephrotoxic effects and myelosuppression	*UL54* DNA polymerase; *UL54* codons 375-540, cross-resistance with GCV.

1.4.2. Current Strategies

The strategy for HCMV infection prevention in seronegative HSCT recipients starts by the selection of blood products from HCMV seronegative donors or use leukocyte-reduced blood products [58, 60, 64, 68]. However, antiviral prophylaxis and viral monitoring with pre-emptive therapy are currently the two strategies used for prevention of HCMV infection and disease in high-risk patients [58, 60, 62, 64, 65, 67]. Overall, intravenous GCV and oral VGCV are preferred as first-line agents for both prophylaxis and preemptive treatment, largely because of their lower toxicities than those of FOS and CDV [68, 74].

HCMV prophylaxis begins prior to transplantations and is continued for a post-transplant period that usually rounds 100 days [19]. It is conceptually attractive because it is simple, may not require viral monitoring, and may prevent both direct and indirect effects of HCMV infection [62, 68]. This therapy has been more used in SOT recipients than in HSCT [17, 36]. Randomized studies have shown that the long-term (3 to 4 months) use of GCV-based prophylaxis, among HCMV-seropositive HSCT recipients, is effective in suppressing early HCMV disease. However, the incidence of bacterial and fungal infections and occurrence of late HCMV disease was increased [85, 86]. A possible mechanism for late HCMV disease in prophylactic therapy is the delayed recovery HCMV-specific T cell responses due to efficient suppression of HCMV by GCV [58]. Furthermore, there are some toxic effects of anti-HCMV drugs that influence the use/efficacy of prophylactic treatment [58, 60, 62]. GCV has been associated with bone marrow depression and this has led to a reduction on the use of GCV-based prophylaxis [17]. The use of other drugs in HCMV prophylaxis has been reduced and FOS has been used for HCMV prophylaxis in pilot studies only [60, 64]. Furthermore, some studies suggest that HCMV prophylaxis, despite useful in some cases, is a high-cost strategy that exposes all patients to drug toxicity, selection of viruses and development of antiviral resistance, and increases the risk of late-onset HCMV infection and disease [87].

Preemptive antiviral therapy is based in two parts: monitoring HCMV infection/reactivation and early intervention in patients when HCMV infection/reactivation is detected [58, 62, 68]. There are several strategies for HCMV monitoring, especially those based in the pp65 antigenemia or HCMV DNA viral load (by real-time quantitative *polymerase chain reaction* (RT-qPCR)), and the sensitivity of tests is crucial for the

effective detection of HCMV prior to disease development [10, 58-60, 62, 64, 68, 79, 88, 89]. Preemptive treatment is applied after the first positive and lasts until day 100 after transplantation or resolution of symptoms or a HCMV-negative test result, which usually result in 2-4 weeks duration of therapy [88, 89]. Then, a maintenance period of therapy is usually used and varies according to the patient (disease response, risk factors-risk of relapse and side effects of the therapy) [17]. Both GCV and FOS can be used for preemptive therapy, with similar efficacy against HCMV, but with different toxicity profiles [60, 62, 65, 68, 90]. The combination of GCV and FOS has been used with high efficacy in patients with a high viral load [90, 91]. Nevertheless, oral VGCV has also been reported in substitution of IV GCV for HCMV infections after HSCT [60, 67, 73]. CDV was found to be effective as second-line therapy especially to rescue patients failing antiviral therapy with GCV, FOS, or both [62, 80, 90] . This approach has the advantage that only patients at highest risk for HCMV disease will get treatment, reducing the risk of side-effects [59, 67]. Preemptive therapy for HCMV has shown to reduce the risk for HCMV disease and improve survival [59, 62, 67]. However, its efficacy is dependent on the sensitivity of the monitoring assay [58].

Preemptive therapy guided by pp65 antigenemia and/or real-time quantitative PCR (RT-qPCR) assays is now more widely used than prophylactic therapy as a strategy against HCMV after allogeneic HSCT because of the bone marrow toxicities of the current anti-HCMV drugs [58, 59]. In fact, PCR-based antiviral therapy was demonstrated to reduce the incidence of HCMV disease and the risk for HCMV associated mortality [59, 88], while that antigenemia-based preemptive therapy was found to be as effective as GCV prophylaxis in preventing HCMV disease [89]. However, RT-qPCR assays have largely replaced the pp65 antigenemia assay for guidance of preemptive antiviral therapy [92].

The treatment for end-organ disease is longer with 2-4 weeks of induction, followed by maintenance therapy for several weeks [17, 68]. The standard recommended therapy for HCMV pneumonia consists in IV GCV or FOS as an alternative agent in combination with high-dose intravenous immune globulin (IVIGs) [17, 58, 68]. For other HCMV diseases, such as Gastrointestinal (GI) or central nervous systems (CNS), IV GCV (or FOS) alone is the most frequent approach [58]. Decreasing immunosuppression is crucial to the treatment of HCMV end-organ disease, however, it must be balanced against the risk of acute GVHD

[17]. Furthermore, the decision to stop therapy or switch to maintenance therapy should be made based on the individual patient response to therapy [58].

1.5. HCMV drug resistance

The emergence of HCMV resistance to one or more antiviral agents is associated with treatment failure and progression of HCMV disease in immunocompromised patients, complicating therapeutic and clinical management [73, 74, 93, 94]. Drug resistance may be suspected if persistent or increasing viral loads or overt HCMV disease occurs after two or more weeks of therapy [19, 68, 74, 93, 95, 96].

The rates of HCMV drug resistance vary widely depending on the type of patients, ranging between 5.0-12.5% for SOT recipients and <4.0% for HSCT recipients. In HIV-infected patients, resistance has been reported in >20% of patients before the availability of highly active antiretroviral therapy (HAART) and in 9% after HAART [19, 40, 74, 79].

In immunocompromised patients, several risk factors have been identified for the emergence of HCMV resistance to antiviral agents [19, 40, 68, 73, 77, 93, 95, 97, 98]. The risk factors may be divided in different categories: 1) patient and disease related factors; 2) treatment-related factors; and 3) viral factors [19, 40, 68, 73, 77, 93, 95, 97, 98]. Regarding the patient and the disease related factors, SOT, underlying disease, type and degree of host immunosuppression and the occurrence of HCMV disease have been described as influencing the risk of drug resistance [40, 95, 96, 98]. Prolonged antiviral therapy, suboptimal antiviral concentrations due to poor compliance or low drug absorption and limited oral bioavailability are important risk factors in the emergence of HCMV drug resistance [40, 46, 68, 73, 96]. The viral factors that appear to play a role in the development of drug resistance are: the establishment of lifelong latency, which allows for later reactivation under the conditions of immunosuppression; the slow lytic replication cycle; higher viral load at the start of the therapy; and the viral DNA polymerase, which has a proofreading function and is responsible for the high fidelity of the replication, which results in a low mutation rate [19, 73].

Actually, it is known that the molecular mechanisms of HCMV resistance to antiviral drugs result from the evolution of single or multiple mutations in the viral phosphokinase (*UL97*) and DNA polymerase (*UL54*) genes that confer various levels of resistance [19, 73, 82, 95, 96]. Moreover, the continuous administration of a drug to which

resistance has been developed can lead to accumulation of multiple drug resistance mutations [74, 96, 99, 100]. The level of resistance of each mutation is expressed as the ratio of the half maximal inhibitory concentration (IC_{50}) of the mutant to that of drug – sensitive wild-type (IC_{50} of mutant/IC_{50} of wild type ratio) [19, 101].

The resistance to GCV/VGCV has been associated with mutations in *UL97*, *UL54*, or both viral genes in laboratory and/or clinical HCMV isolates [78, 97, 102]. However, mutations in *UL54* DNA polymerase are less common than mutations in the *UL97* gene [82, 103]. Despite of the majority of *UL54* mutations that confer resistance to GCV/VGCV also confer it to CDV and/or FOS [19, 82, 96, 99, 103], there are also mutations *UL54* that confer only to the GCV/VGCV - Table I [98, 104, 105]. Moreover, resistance to FOS or CDV in laboratory and/or clinical isolates has been mapped to amino acid substitutions in pUL54. Cross-resistance between GCV, CDV and FOS has been reported in pUL54, in laboratory and/or clinical isolates, with both genotypic and phenotypic resistance - Table I, Attachment I and II [19, 78, 97, 102].

HCMV drug-resistance can be determined by phenotype or genotype assays [19, 40, 75, 95, 98, 106]. Phenotype assays are time-consuming whereas genotype assays are faster and easier to perform, however, the mutations detected through genotype assay may be hard to interpret [19, 40, 75, 97, 98, 106, 107]. Thus, the discrimination between polymorphisms (sequence variants not reducing drug susceptibility) and resistance-conferring mutations is determined by recombinant phenotyping (marker transfer) assays [101, 106].

1.5.1. *UL97* Mutations

UL97 is on the position 97 of the unique long region of HCMV genome and is a β2 delayed early gene encoding for the viral phosphokinase pUL97 [19, 55, 97]. The pUL97 amino acid sequence contains serine/threonine kinase functional motifs conserved in different host species including yeast, bovine, rat and human [19, 55]. This protein undergoes auto phosphorylation, which is required for subsequent phosphorylation of serine and threonine residues of various cellular and viral proteins, and is required for efficient HCMV replication [19, 77, 98].

Structurally, pUL97 can be divided into different conserved functional regions: I, II, III, VIB, VII, VIII and IX [40, 55, 77, 98]. Codons defined for each regions are as follows: region I, 338 to 345; region II, catalytic lysine 355; region III, glutamate 380; region VIB, 453 to 462; region VII, 481 to 483; region VIII, 520 to 527; and region IX, 574 to 579 [19, 55]. While region I is responsible for adenosine triphosphate (ATP) binding, regions II, III, VIB and VII are involved in the phosphate transfer and region IX is essential for substrate binding - Figure 8 [55, 95].

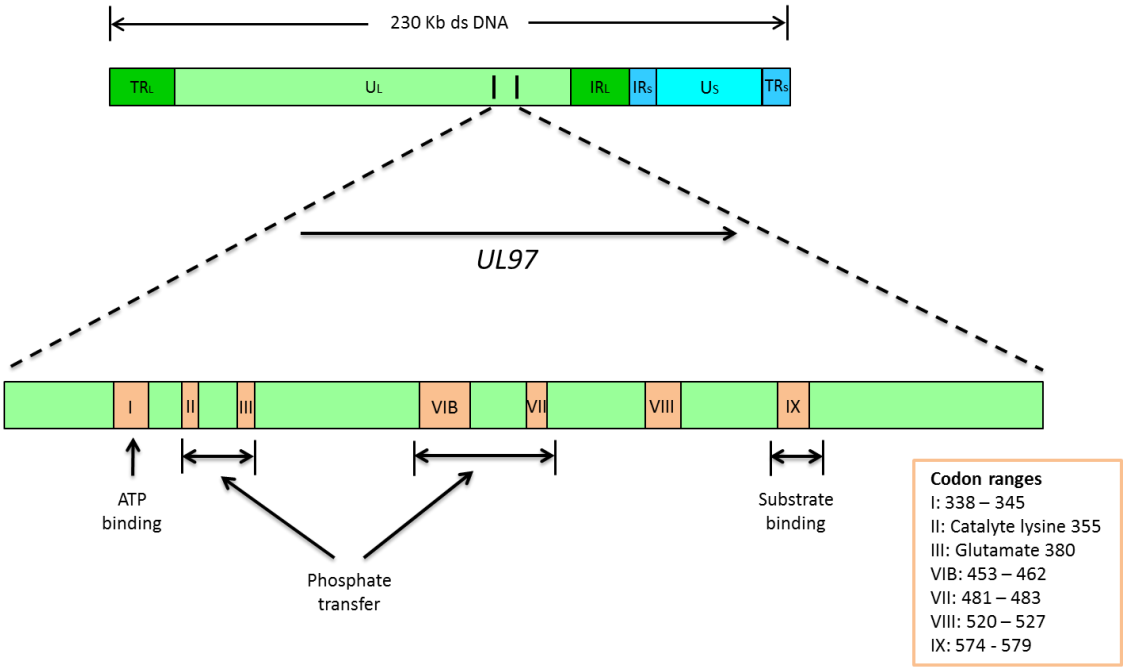


Figure 8: *UL97* structure and functional domains. Adapted from [19, 55, 77, 95].

pUL97 kinase has a crucial role in the phosphorylation of the GCV, which is necessary for generation of its active forms and consequent inhibition of viral DNA polymerase [40, 55]. Hence, mutations in *UL97* that impair this phosphorylation in virus-infected cells are the preferred mechanism of viral resistance [58, 62]. *UL97* mutations, which are clustered over a relatively small genomic region, confer resistance to GCV/VGCV, but do not affect susceptibility to FOS or CDV [40, 55, 74].

Mutations that confer resistance to GCV/VGCV are typically amino acid substitutions or short (1- to 17-amino-acid) in-frame deletions that change the ATP binding site or the phosphate transfer site altering its ability to phosphorylate GCV, but without impair its function in viral replication [19, 55, 75, 95, 98, 108]. Approximately 95% of

GCV/VGCV-resistant HCMV strains contain one or more mutations in *UL97* [68, 78, 81, 94, 108-110]. GCV/VGCV resistance mutations are strongly clustered at codons 460 (region VIB), 520 (region VIII), and 590-607 (nonfunctional region), with three specific codons (460, 594, and 595) accounting for approximately 70% of GCV/VGCV-resistant HCMV strains [19, 40, 55, 77, 81, 108-110]. Mutations at codons 460 and 520 are located at conserved kinase domains more likely to be critical to overall kinase function, which probably explains the more limited range of mutations observed at these sites [19, 55]. The codon segment 590–607 is dispensable for viral replication. Nevertheless, mutations in this region impair the recognition of GCV as a substrate while preserving the normal biological functions [19, 55]. In fact, in this region it may be observed a large variety of amino acid substitutions and deletions that confer different degrees of resistance – Attachment I [19, 55, 101]. Furthermore, the impact of each mutation in GCV/VGCV-resistance is different and the GCV/VGCV IC_{50} has been shown by recombinant phenotyping for a great majority of them [95, 97].

By revising the literature regarding the *UL97* mutations in immunocompromised patients, including transplantation recipients and patients with AIDS, it was found that the most common *UL97* mutations associated with GCV/VGCV resistance are M460I/V, H520Q, C592G, A594V, L595F/S/W and C603W [107-111]. In fact, one or more of these canonical mutations appear in over 80% of GCV/VGCV-resistant isolates [55]. All of these mutations have been described by recombinant phenotyping to confer a 5- to 16-fold increased GCV/VGCV IC_{50} [77, 97, 101, 108, 112], except C592G, which has been referred as preferentially selected in situations where the virus is exposed to low GCV concentrations such as when oral therapy is insufficiently absorbed or bioavailable – Attachment I [55, 77, 107, 108].

Although the dominance of these seven canonical mutations as laboratory markers of GCV/VGCV resistance is confirmed by all recent clinical studies, a variety of uncommon resistance mutations are known to occur in different *UL97* codons [77, 98, 107]. Amino acid substitutions F342S, V356G, L405P, D456N, M460T, V466G, C480R C518Y, P521L, C592F, A594E/G/P/T, L595T, E596G/Y, K599T, C603R, C607T/Y, I610T, A613V and the deletion variants (del355, del591-594, del590-600, del591-607, del595, del595-603, del601-603, del617) are uncommon resistant mutations that were reported to confer at least a 2-fold reduction in susceptibility to GCV/VGCV in phenotypic

assays [96, 101, 108, 113-124]. Some of these mutations (L405P, V466G, A594E/P/T, L595T, E596G, I610T and A613V) confer low-grade resistance (2-4x increased IC_{50}) , while others (F342S, del355, V356G, D456N, M460T, C480R, C518Y, P521L, del591-594, del590-600, del591-607, C592F, A594G, del595, del595-603, E596Y, K599T, del601-603, C603R, C607T/Y, and del617) confer a moderate to high GCV/VGCV resistance (5-32x increased IC_{50}) [95-97, 101, 108, 113-125]. Nevertheless, reductions in susceptibility of <2-fold have been reported to be clinically significant [97, 108]. For example, the mutations, del600, C603S, C607F and E655K have been shown to confer a <2-fold reduction in phenotypic assays [19, 96, 101, 108, 126]. Despite the lack of a clear resistance phenotype, the amino acid substitutions, C603S and C607F, occur at a conserved site where other mutations have been shown to be associated with ganciclovir/valganciclovir resistance. All of these mutations have been shown to be associated with GCV/VGCV resistance and have been also observed in patients failing treatment or prophylaxis – Attachment I [97, 108, 126].

In addition, there are also other mutations (M460L, del590-603, del594-595, del596, G598S, K599E, del601 and T601M) that appear to confer significant GCV/VGCV resistance based on recombinant phenotyping, but do not have an IC_{50} value reported in comparison to a wild-type reference – Attachment I [127-133].

Many other *UL97* sequence changes found in clinical isolates have not been yet phenotyped by marker transference, mainly because the size of the HCMV genome makes its site-specific mutagenesis technically nontrivial [101]. So far, despite have been observed in patients failing treatment or prophylaxis, there is no published data to characterize the phenotype of the mutations E596D, G598V, K599M, C603Y and C607S, which occur at the same position as a confirmed resistance-associated amino acid substitutions and were found in the HCMV *UL97* gene together with the mutations conferring resistance to GCV/VGCV [97, 129]. Moreover, V498I, A590T, A591D, N597I, C606D and deletion variants del600-601, del601-602 and del597-603 are other mutations of unknown phenotype but also observed in patients failing treatment or prophylaxis and all, except the del597-603, were found in the HCMV *UL97* gene together with the mutations conferring resistance to GCV/VGCV [73, 97, 129, 134, 135].

UL97 natural polymorphism mainly focused on codon range 460-607, where all known GCV/VGCV resistance mutations are located [55]. Some amino acid positions of *UL97* can be involved in both natural polymorphism and antiviral resistance [136]. For

example, K599T was previously reported to induce GCV/VGCV resistance [115] whereas K599R has been assessed as a polymorphism [101]. Similarly, V466G, but not V466M, confers resistance to GCV/VGCV [101, 122, 136]. Despite of amino acid substitution V466M being considered, by various authors, as a natural polymorphism, this mutation has also been observed in patients failing treatment or prophylaxis [97, 137]. Other sequence changes as H469Y, A478V, A588V, L600I, M615V, G623S, T659I, and V665I were found to confer no significant GCV/VGCV resistance [55, 101, 122]. Nevertheless, some natural polymorphisms may modulate the drug-resistance level provided by other mutations [100, 136]. Indeed, it has been evidenced by marker transfer that D605E change does not confer any GCV/VGCV resistance and did not affect the resistance conferred by several common *UL97* resistance mutations [55]. Nevertheless, some authors suggest that the D605E mutation could "partially or totally compensate" for the effect of the GCV/VGCV resistance, conferred by the common mutation M460V [100, 106]. Additionally, a study using *vaccinia* recombinants viruses concluded that D605E partially reversed the GCV/VGCV resistance conferred by the unusual A594P mutation [106, 113]. Similarly, N510S mutation has been identified in phenotypically GCV/VGCV sensitive HCMV clinical isolates and it has been also shown not to confer significant GCV/VGCV resistance [101, 136]. This N510S mutation has also been evidenced in a clinical isolate harboring the 591–594 deletion associated with resistance, leading to the hypothesis that N510S mutation may be involved in the modulation of GCV/VGCV resistance induced by the 591–594 deletion [136]. However, Chou and its collaborators showed that the level of GCV/VGCV resistance of 591–594 deletion was not higher than the clinical isolate harboring the 591–594 deletion alone [108].

Finally, there are some mutations described *in vitro* studies (G340V, A442V, L446R, and F523C) that lead technically to GCV/VGCV resistance, but are not expected to occur in clinical isolates since such mutations impair biological function by the loss of autophosphorylation and thus promote a severe growth deficiency [19].

1.5.2. *UL54* Mutations

The *UL54* gene is a β2 delayed early gene located on the position 54 of the unique long region of HCMV genome which encodes for the HCMV DNA polymerase (pUL54), a

relatively large protein (140 kDa) composed by 1242 amino acids [97]. Both DNA and amino acid sequence analysis revealed that the *UL54* gene has significant homology to the *pol* genes of other herpesviruses, such as herpes simplex virus type 1 (HSV-1) and EBV, but also with DNA polymerases encoded by a wide range of organisms [81, 97].

There are two extremely important functions in viral polymerases that share strong homology in different species: the 3´-5´ exonuclease activity (Exo I through III) and polymerization (I through VII). Although it has been proposed that the Exo I to III motifs constitute the 3´ to 5´ exonuclease site of the enzyme, enzymatic analyses of purified mutated HCMV DNA *pol* proteins (L501F and K513N) suggest that the N-terminal portion of a Delta-region (∂-region) C also participates in this enzyme activity [19, 77]. This ∂-region C is also found among herpesvirus polymerases and is also shared with some mammalian and yeast delta DNA polymerases [19, 77]. Despite the specific role of each region, it has been shown that the two enzymatic functions may not behave independently in herpesviruses and may, indeed, overlap [77]. In fact, there is an overlap of Exo II with polymerization region IV and of Exo III with ∂-region C - Figure 9 [19].

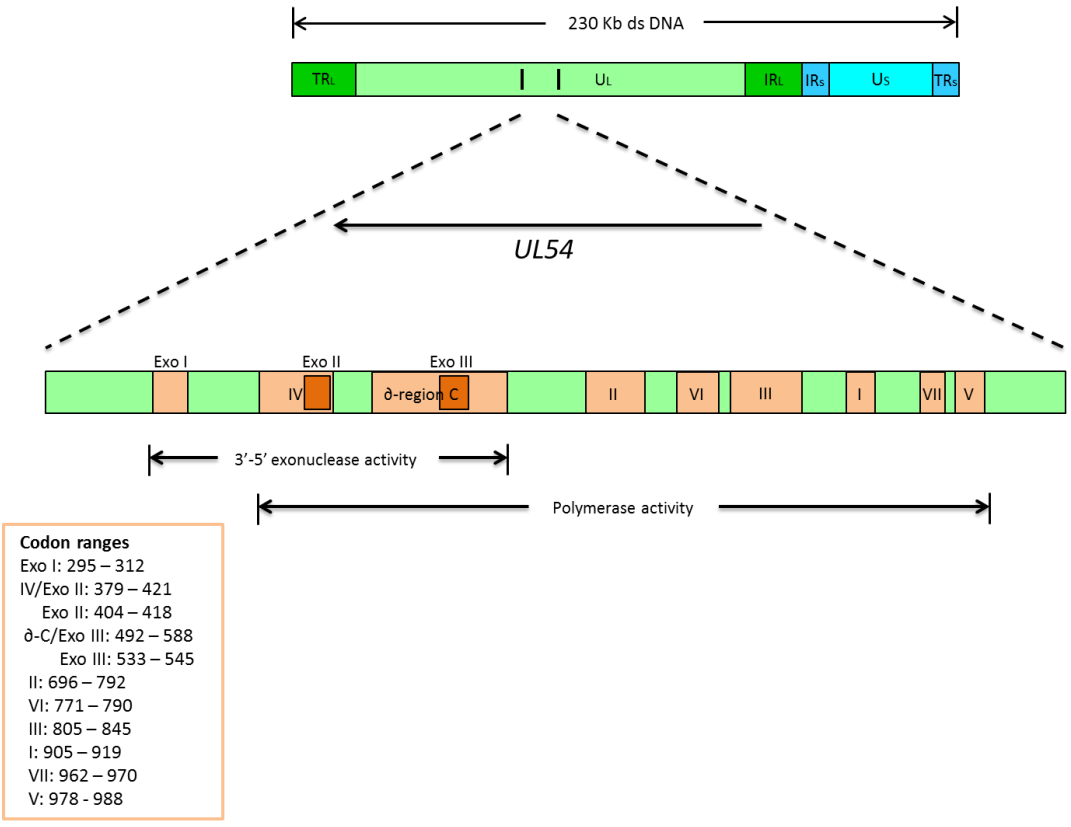


Figure 9: *UL54* structure and functional domains. Adapted from [19, 77, 95].

pUL54 is the central enzyme involved in viral DNA replication and the primary drug target for current therapies (GCV/VGCV, FOS and CDV) [75, 76, 97]. The three possible mechanisms of resistance that have been postulated for the substrates (GCV triphosphate and CDV diphosphate) of DNA *pol* enzyme are: 1) a decreased affinity of the enzyme for the inhibitor; 2) a decreased selective incorporation of the inhibitor into the elongating DNA chain; or 3) an enhanced selective excision from the DNA chain of the incorporated inhibitors [19, 77]. In case of FOS, which is not incorporated into the elongating DNA, only the first mechanism of resistance (decreased affinity of the enzyme) would apply [19, 77].

UL54 mutations, which are clustered over a much larger area, can confer resistance to all current dugs and may emerge after prolonged therapy to increase the level of resistance conferred by a pre-existing *UL97* mutation [87, 98]. Although is uncommon, it has been reported the appearance of a resistance *UL54* mutation in the absence of a *UL97* mutation [19, 78, 138, 139]. Indeed, the combination of mutations in pUL97 and pUL54 resulted in greater reduction in susceptibility to GCV/VGCV than in isolates with only one mutation [112, 140, 141]. For example, the *UL54* mutations A809V, T813S, and G841A, which showed similar phenotypes of low-grade GCV/VGCV resistance, when combined with the *UL97* C592G mutation, result in a significant increase in GCV/VGCV resistance [141]. Similar results were found for the combination of *UL54* del981-2 and *UL97* C592G, and *UL54* L773V and *UL97* C607Y, conferring higher GCV/VGCV resistance than either mutation alone – Attachment I and II [112, 140].

The majority of resistance mutations in *UL54* occur within the conserved regions of homology and many occur at amino acid residues highly conserved among enzymes from herpesvirus and from unrelated organisms [19]. Thus, the particular resistance phenotype largely correlates with its location within the genome [40, 75, 77, 78]. Moreover, multiple *UL54* mutations are additive and cause resistance to multiple antiviral agents [78]. Mutations that confer resistance to GCV/VGCV and CDV are most often found within the exonuclease domains (conserved region IV and the N-terminal extremity of ∂-region C) and region V (C-terminus), whereas mutations located within the C-terminal extremity of ∂-region C and within or next to conserved regions II and VI, seem to be mostly involved in FOS resistance [40, 77, 78, 98]. Deletion of codons 981-982 in region V causes resistance to GCV, CDV, and FOS [40, 77, 78], while mutations within conserved region

III can be associated with resistance to any single agent or combination of agents – Attachment II [77]. No drug resistance mutations have so far been detected in regions I and VII [19]. Conversely, the majority amino acid polymorphisms that are not associated with drug resistance occur at residues outside the conserved regions [19, 78]. However, polymorphisms in this gene are relatively common which makes it more complicated to distinguish them from true resistance mutations. In fact, two polymorphisms (E506K in ∂-Region C and R785S in region VI) may be associated with resistance since they lay within conserved regions [139]. Although position 506 lay within a conserved region, there is high amino acid variability at this position amongst the herpesvirus DNA polymerase, which supports the theory that E506K may be a natural variant [139].

Several studies have documented the emergence of a wide variety and distribution of mutations (amino acid substitutions and deletions) in drug-resistant HCMV clinical isolates from immunocompromised patients including transplantation recipients and patients with AIDS – Attachment II [82, 96, 103, 105, 116, 123, 126, 134, 138, 140, 142-150]. The D542E and K805Q are the single mutations which confer resistance only to CDV [96, 116, 148, 151, 152]; while L802V, P829S, L862F and L957F, are the mutations that confer resistance only to GCV/VGCV [98, 105]; and the mutations that confer resistance just to FOS are N495K, S585A, D588E, F595I, A692S, T700A, V715M, E756D/Q, W780V, T838A, M844T and V946L [75, 96, 98, 105, 116, 138, 140, 143, 149-151, 153-155]. Nevertheless, the majority of the *UL54* mutations that confer resistance to GCV/VGCV also confer it to CDV and/or FOS, therefore cross-resistance between GCV/VGCV and CDV and/or FOS has been reported for several amino acid substitutions and two deletions [19, 78, 95-97, 102]. However, cross-resistance between GCV/VGCV and CDV has been reported more frequently than between the GCV/VGCV and FOS [19, 78, 95-97, 102]. Among the most frequent mutations associated with drug resistance are T503I, P522S, L545S and A987G [73, 74, 82, 96, 116, 134, 139, 140, 145, 153, 156] which confer resistance to GCV and CDV; V781I, L802M, and A809V which confer resistance to GCV and FOS [74, 96, 104, 111, 116, 134, 140, 145, 153, 155-157]; and V715M which confers resistance to FOS [94, 96, 134, 140, 156, 158, 159]. In addition, there are some frequent mutations that confer resistance to all three drugs (D588N, Q578H and A834P) [74, 82, 96, 103, 140, 145] Although the dominance of these mutations, a variety of uncommon resistance mutations have also been reported in both laboratory and clinical strains – Attachment II [19, 78, 95-97, 102].

As in *UL97*, the impact on drug resistance of each mutation in *UL54* is variable and has been demonstrated by several recombinant phenotyping studies– Attachment II. The D301N, N408D/K/S, N410K, F412C/L/S/V, D413A/E/N, P488R, K500N, L501I, T503I, K513E/N/R, L516R, I521T, P522A/S, V526L, C539G/R, L545S/W, A987G and del524 are reported to confer ≥ 2-fold reduction in susceptibility to GCV and CDV [19, 95, 97, 105, 126, 143, 144, 151, 160-162]. With except for D413E, K500N and V526L all other mutations exhibit greater resistance to CDV than to GCV [95, 97, 105, 126, 143, 144, 151, 160-162]. Similarly, there are mutations with cross-resistance (≥2-fold reduction) to GCV and FOS (D515E/Y, V776M, V781I, V787A/L, L802M, A809V and G841S), and the majority exhibit a greater resistance to FOS than to GCV [95, 97, 105, 122, 141, 146, 151, 155]. The Q578H, D588N, E756K, L773V, V812L, T813S, A834P, G841A and del 981–982 confer a ≥2-fold reduction for all three drugs, however the del 981–982 exhibit a greater resistance to GCV than to FOS and CDV – Attachment II [97, 105, 140, 141, 143, 160, 161, 163].

Considering the mutations in *UL54* that confer higher rate of GCV/VGCV-resistance, D413A, L501I, K513N and deletion of codons 981 to 982 have been associated with a 6- to 8-fold increased resistance [19, 97, 112, 142, 143, 151, 163, 164]; for CDV-resistance, N408K, F412C/S/V, D413A/N, K513N/R, C539R, D542E and A987G have been associated with a 10- to 21-fold decrease in CDV susceptibility [19, 95, 97, 105, 148, 151, 160, 161]; and for FOS, D588N, V715M, E756K, L802M, A809V, T821I and A834P seem to confer 5.5- to 21-fold increased resistance [19, 95, 97, 105, 141, 143, 151, 152, 160]. In addition, there are some mutations that confer a <2-fold reduction in susceptibility to HCMV-antiviral drugs in phenotypic assays and that have been found in both laboratory and clinical isolates (A505V, T552N, Q578L, I726T/V, W780V, L802V, T821I, M844V and L872F) – Attachment II [97, 98, 105, 140, 146, 149].

Despite of some *UL54* mutations have little direct impact on HCMV susceptibility to drugs, they may increase the effect of other mutations, either by directly enhancing resistance or indirectly by increasing viral fitness (viral replication kinetics) [97, 98, 105, 152, 160]. For example, the HCMV DNA polymerase mutation K805Q improved the viral fitness of the mutation T821I associated with high levels of resistance to FOS [152]. It has also been shown that the N408K mutation, in combination with A834P, partially reconstituted the replication impairment of recombinant virus containing only A834P

[160]. This suggests that perturbation of both DNA polymerization (A834P) and exonuclease (N408K) activities contributes to antiviral resistance and altered replication kinetics in these mutant strains [160]. Additionally, the combination N408K+A834P also increased GCV/VGCV and CDV resistance compared to the levels of resistance of the virus containing only the A834P mutation [160]. Others combinations, such as N408D/L957F and L545S/P829S showed FOS resistance whereas the single mutations did not [98, 105]. The mutation L957F is also apparently able to modulate the FOS resistance level conferred by mutation T552N alone [105]. Some studies have suggested decreased viral fitness of HCMV strains with drug-resistant mutations (K513N, D588N, T838A,) [155, 164] and a reduction of infectious virus yield of N495K, T700A and V715M [154, 159]. This is expected to be caused by the occurrence of these mutations within conserved regions and also because resistance to FOS is associated with a slower replication of HCMV – Attachment II [159].

Moreover, there are some natural polymorphisms on the sequence of *UL54* of which the impact on resistance is unknown. For example, the P522L polymorphism, located at the same position as confirmed resistance-associated mutation (P522S), has also been observed in patients failing treatment or prophylaxis [97]. In addition, E315D, D879G and A972V polymorphisms have also been observed in multiple patients failing treatment or prophylaxis [73, 97].

Furthermore, there are several other amino acid substitution (i.e. M393R/K, L501F, L516M, P608S, T610M, A614S, G629S, I722V, Y751H, S880L, S897P and R1052C) found in clinical isolates that have not been phenotyped by marker transfer yet [73, 102, 104, 116, 138, 145, 165]. A number of other mutations observed after *in vitro* or *in vivo* drug exposure have not been confirmed, and they illustrate the potential diversity and distribution of pol mutations, which may not be the same in laboratory strains as in clinical isolates [19].

Chapter 2

Objectives

Important studies showed that HCMV drug resistance mutations are associated with a poor outcome and with a significant patient morbidity and mortality. Indeed, the characterization of HCMV drug resistance mutations has contributed to the progress of the HCMV therapy and to understand the basic biological functions of virus-encoded products that serve as antiviral drug targets. Thus, the fast detection of these HCMV drug-resistance strains, directly from patients, is an important issue, improving an early management of patients with alternative treatments.

2.1. Main Objective

The main objective of this dissertation was to characterize HCMV mutations on allogeneic HSCT patients and correlate with patient's response to treatment.

2.2. Secondary Objectives

For this dissertation we have defined the following secondary objectives:

1. Characterize HCMV mutations in *UL97* and *UL54*;
2. Correlate HCMV mutations with antiviral treatment response;

Chapter 3

Patients and Methods

3.1. Type of Study and Population

We have performed a retrospective study in a cohort of patients with different haematological malignancies submitted to allogeneic Hematopoietic Stem Cell Transplant (allo-HSCT), between 2010 and 2014, at the *Bone Marrow Transplant Service* from Portuguese Institute of Oncology of Porto *(IPO Porto)*. Cases were selected for mutation analysis if had more than one of the following: 1) reactivation prior 30 days after transplant; 2) viral load at first positive detection $>10^4$ or > 5 positive cells; 3) more than 1 month of HCMV positivity; 4) under preemptive treatment for more than 2 weeks with no significant decrease or increase of HCMV.

This study was approved by the local ethical committee (CES IPO: 73/2015) and did not interfere with routine procedures decided by clinicians – Attachment VI.

3.2. HCMV infection monitoring

Anti-HCMV prophylaxis was not performed. However, herpesvirus prophylaxis was performed with acyclovir (5 mg/kg/dose, every eighth hours) for four weeks. All patients undergoing allogeneic HSCT were monitored after transplant for active HCMV infection using either: 1) pp65 antigenemia (pp65-Ag) assay, based on detection of HCMV pp65 antigen in infected cells using the C10/C11 monoclonal antibody cocktail (IQ Products®, Groningen, Netherlands); or 2) when White Blood Cells (WBC) count was <1000/mL by a real-time quantitative *polymerase chain reaction* (RT-qPCR), which amplifies a region from the exon 4 of the HCMV major immediate early antigen (MIEA), using the *Q-CMV Complete Kit* (EliTech Group®, Puteaux, France). Briefly, the monitoring was performed in peripheral blood samples starting at the day of the transplant

with bi-weekly analysis up to 100 days after transplant, weekly up to 180 days, bi-monthly up to 1year, and after the first year on routine evaluations.

3.3. Management of post-allo-HSCT HCMV infection

All patients were followed post-transplant using a preemptive strategy for HCMV infection/disease prevention. Preemptive therapy was initiated after two consecutive positive results for HCMV, either by pp65-Ag (≥ 1 antigen-positive cell per $5{,}0x10^4$ polymorphonuclear leukocytes (PMNL)) or by RT-qPCR (viral load ≥1000 copies/mL). Preemptive anti-HCMV therapy is initiated with either Ganciclovir (GCV) or oral Valganciclovir (VGCV). VGCV is used as first-line treatment at a dose of 900 mg twice a day (12h/12h) during 2-3 consecutive weeks. In the context of gastrointestinal intolerance, oral VGCV is replaced by GCV as second-line treatment: GCV at a dose of 5 mg/kg twice a day (12h/12h) by 2-3 consecutive weeks. Patients with severe VGCV or GCV-associated complications or with no laboratory response were treated with intravenous foscarnet (FOS) as third-line treatment, at a dose of 60 mg/mg/8h. Antiviral doses are closely monitored according to patients` renal function. After two consecutive negative results for HCMV, patients start maintenance therapy with VGCV 450 mg once a day; or GCV 5 mg/kg once a day during 5 days per week; or FOS at a dose of 90 mg/kg once a day during 5 days per week until day +100 or day +180 in high risk patients.

3.4. Data collection and analysis

The patient's demographic characteristics (gender, age), clinicopathological data (underlying diseases, stem cell source, HLA status, conditioning regimen and HCMV donor/recipient serostatus) and treatment information (strategy: prophylactic or preemptive; type of antiviral: VGCV, GCV, FOS or CDV; dose and duration) of 22 patients were collected from individual clinical records by a clinician and are described in Table IV.

3.4.1. Variable definitions

Hematological diseases were classified into the following categories: acute leukemia; chronic myeloproliferative disorders; chronic lymphoproliferative disorders; Myelodysplatic/Myeloproliferative diseases; aplastic anemia; and others.

HCMV infection was defined as a positive result in either pp65 antigenemia (≥ 1 antigen-positive cell per $5{,}0x10^4$ polymorphonuclear leukocytes) or RT-qPCR (viral load in whole blood sample ≥100 copies/mL). Time to infection (TTI) was defined as the difference between the day of allogeneic HSCT and the day of first HCMV positive result. Early HCMV infection was defined as occurring before 100 days after allogeneic HSCT, whereas late HCMV infection was defined as occurring after 100 days after allogeneic HSCT. The end of an infection episode was defined as the first of two consecutive days with negative result. Recurrent HCMV infection was defined as a new detection by pp65 antigenemia assay at least four weeks after the clearance of the preceding episode. If a positive result was obtained within the four weeks after clearance, it was considered to be the same episode of the previous HCMV infection. The duration of infection (DOI) was defined for the first HCMV infection episode as the difference between the day of the first positive HCMV result and the day of the last positive HCMV result (that is, the positive result preceding four consecutive negative results during biweekly monitoring).

3.5. HCMV genotyping

3.5.1. Sample processing and nucleic acids extraction

Peripheral blood samples collected in ethylenediaminetetraacetic acid (EDTA)-containing tubes used for HCMV diagnosis were used for Nucleic Acid (NA) extraction and genotyping of HCMV.

Nucleic acids extraction was performed using *Magna Pure Compact Nucleic Acid Kit* (Roche, Indianapolis, USA) according to manufacturer`s instructions. To validate the extraction method, nucleic acids quality was assessed by measuring the absorbance at 260/280nm using an UV/Visible spectrophotometer and its purity assessed by the ratio of

the values of absorbance at 260/280nm. The presence of genomic DNA was tested with a PCR protocol for amplification of cyclin D1 gene [166].

3.5.2. HCMV genotypic antiviral resistance

HCMV genotypic antiviral resistance test was based on the analysis of *UL97* and *UL54* mutations. The protocols for amplification of *UL97* and *UL54* were adapted from published manuscripts and given by Professor David Boutolleau [73, 136]. The amplification and sequencing conditions, which were used to characterize the *UL97* and *UL54* mutations, are reported bellow.

3.5.2.1. Amplification of HCMV full-length *UL97* gene

The protocol for *UL97* amplification is based in a Nested-PCR reaction. The first PCR, which amplified a fragment of 2389 bp, was performed in a 50 µL of PCR reaction mix containing 1x buffer, 0.3 mM dNTPs, 0.3 µM of forward and reverse outer primers (Table II), 1U DNA polymerase (KAPA Hifi HotStart DNA Polymerase, KAPABIOSYSTEMS, Boston, Massachusetts, United States) and 2 µL of test DNA. The amplification conditions were as follows: initial denaturation at 94ºC for 5 min, 40 cycles of amplification followed by denaturation at 98ºC for 20s, annealing at 60.4ºC for 20s, and extension at 72ºC for 60s, then final extension at 72ºC for 5 min.

The second PCR amplifies a 2288 bp fragment of *UL97* using the inner primers (Table II) in a 50 µL reaction mix with the same conditions as the first PCR and with 0.2 µL of the first PCR product. The amplification conditions were: initial denaturation at 94ºC for 5 min, 30 cycles of amplification followed by denaturation at 98ºC for 20s, annealing at 58.7ºC for 20s, and extension at 72ºC for 60s, then final extension at 72ºC for 5 min. This PCR amplifies the full-length *UL97* sequence, including all of the known GCV/VGCV-resistance mutation sites from codons 342-708.

PCR products were analyzed by electrophoresis with a 0.5% (w/v) agarose gel stained by ethidium bromide and visualized under UV light – Figure 10. PCR products resulting from the nested PCR were purified using the Qiagen QIAquick Gel Extraction Kit (QIAGEN GmbH, Hilden, Germany).

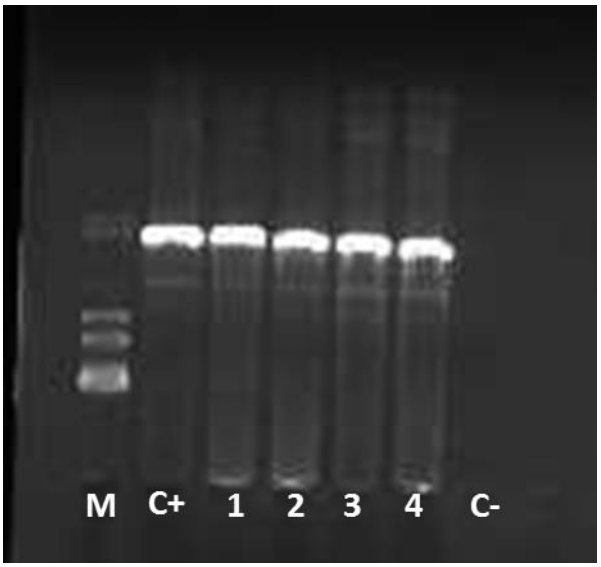


Figure 10: Second PCR product with 2288 bp indicates the presence of the amplification product of HCMV *UL97* gene. 1-4: samples; M-marker; C+: Positive Control; C-: Negative Control.

3.5.2.2. Amplification of HCMV partial *UL54* gene

The protocol for *UL54* amplification is also based in a Nested-PCR technology. The first reaction was performed in a 25 µL of reaction mix containing 1x buffer rich GC, 0.2 mM dNTPs, 0.3 µM forward and reverse outer primers (Table III), 1U DNA polymerase (KAPA Hifi HotStart DNA Polymerase, KAPABIOSYSTEMS, Boston, Massachusetts, United States) and 1 µL of DNA. The amplification conditions were: initial denaturation at 94ºC for 5min followed by 40 cycles at 94ºC for 20s (denaturation), 60.4ºC for 20s (annealing), and 72ºC for 60s (extension), and a final extension at 72ºC for 5 min. This PCR reaction amplifies a fragment of 2576 bp of the HCMV *UL54*.

The second PCR was performed using the inner primers (Table III) in similar conditions to the first PCR and 0.1 µL of first round PCR product. The amplification conditions were: initial denaturation at 94ºC for 5 min, followed by 30 cycles of 94ºC for 20s (denaturation), 60.4ºC for 20s (annealing), and 72ºC for 60s (extension), and a final extension at 72ºC for 5 min. This second PCR amplifies a fragment of 2408 bp of *UL54*, which includes known antiviral-resistance mutations sites from codons 269-1070. PCR products were analysed by electrophoresis in 0.5% (w/v) agarose gel stained with ethidium bromide and visualized under UV light – Figure 11.

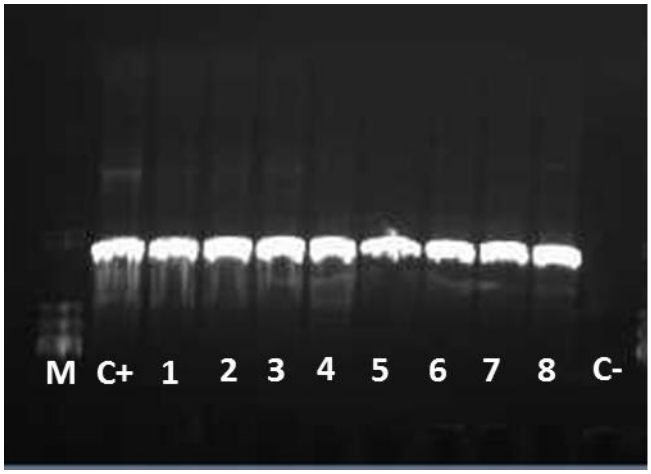


Figure 11: Second PCR product with 2408 bp indicates the presence of the amplification product of HCMV *UL54* gene. 1-8: samples; M-marker; C+: Positive Control; C-: Negative Control.

3.5.2.3. Sequencing of HCMV *UL97* and *UL54*

UL97 and *UL54* products were sequenced using overlapping primer pairs with the *Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit* (Applied Biosystems, Courtaboeuf, France) and analyzed with the automated sequencer ABI 3730 Genetic Analyzer (Applied Biosystems, Foster City, USA). All primers for amplification and sequencing are listed in Table II and III.

Table II: Primers used for amplification and sequencing of HCMV full-length *UL97* gene.

Target gene	Function	Name	Sequence (5´→3´)
UL97 (2389 bp)	First-round PCR (outer primers)	*UL97*-F1	F:CGACGCCGTCTAACAGGTAT
		UL97-R1	R: CTCATCGTCGTCGTAGTCCA
UL97 (2288 bp)	Second-round PCR (inner primers)	*UL97*-F2	F: TCACGCCTCTGTTCAGATTTT
		UL97-R2	R: CGGTGGGTTTGTACCTTCTC
UL97	Sequence reaction	*UL97*-A	R: TCGTACTCGAACGACCACAT
		UL97-B	F: GAAACTTCGGCCATGTGGT
		UL97-C	R: CAGACCTCGCCGAAGGAG
		UL97-D	F: GACATGAGCGACGAGAGCTA
		UL97-E	R: AAAAGCCCAGCACGTTACC
		UL97-F	F: CTACGGCGTTATTGCATGTC
		+*UL97*-F2 and *UL97*-R2	

Table III: Primers used for *UL54* gene amplification and sequencing.

Target gene	Function	Name	Sequence (5´→3´)
UL54 (2576 bp)	First-round PCR (outer primers)	*UL54*-1	F: ATCTGCTGGAGCAGGGTTTT
		UL54-11	R: CCAATCGCTTAATGACGGCA
UL54 (2408 bp)	Second-round PCR (inner primers)	*UL54*-2	R: TTGACGGTACAGCGAGATGT
		UL54-3	F: GCGTCGACTTGTGATATCGA
UL54	Sequence reaction	*UL54*-4	R:ATCCTCAAAGAGCAGCGAGA
		UL54-5	F:GCGCGGTTCATCAAAGACAA
		UL54-6	R:AAAGCGGACAAACACGCTGT
		UL54-7	F: TGGCTAAAATTCCGTTGCGG
		UL54-8	R: ACCTTTGCTGTAGTGGTTGG
		UL54-9	R:GCATACAGGGTACATGTCGAT
		UL54-10	F: TCGGCTTCTCACAACAATC
		+*UL54*-2 and *UL54*-3	

3.6. HCMV sequence analysis

The sequence data was observed using the *4Peaks for Mac OS X 10.3* version 1.7.2 freeware program developed by A. Griekspoor and Tom Groothuis and available at http://nucleobytes.com/index.php/4peaks.

The analysis of the sequence data from *UL97* and *UL54* of all patients was performed by the *MRA – Mutation Resistance Analyzer* online tool (http://www.informatik.uni-ulm.de/ni/mitarbeiter/HKestler/mra/app/index.php?plugin=form) from the *Universität Ulm, AG Bioinformatics and Systems Biology, Institute of Neural Information Processing, Institute of Virology*. This tool compares the sequence results with the reference sequences: *UL97* from Merlin strain (GenBank accession No: AY446894.2); and *UL54* from TB40/E strain (GenBank accession No: ABV71585.1) – Figure 12 and 13, and Attachments III and IV.

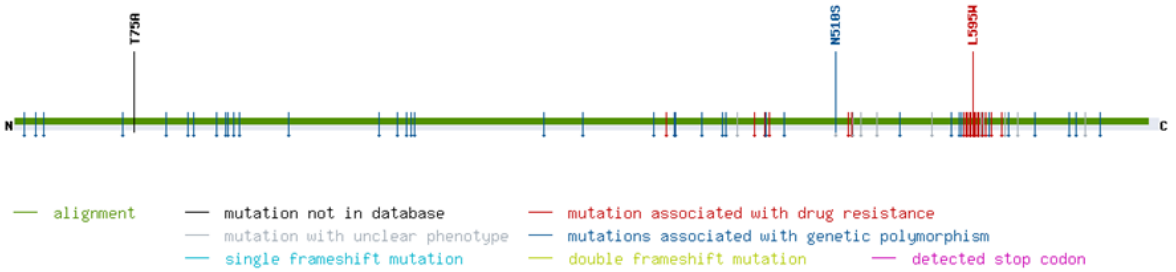


Figure 12: *UL97* sequencing analysis of codon 1 until 707 in comparison to Merlin strain. The mutations found were: T75A, N510S and L595W.

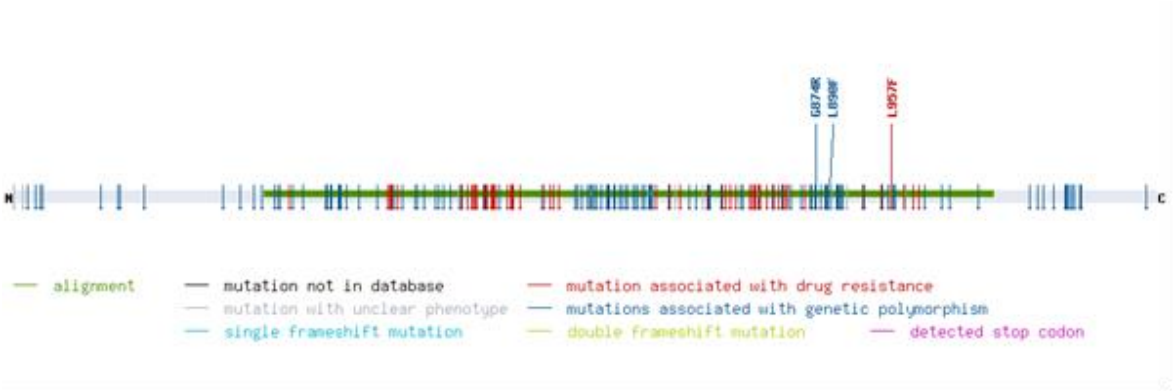


Figure 13: *UL54* sequencing analysis of codon 272 until 1069 in comparison to TB40/E strain. The mutations found were: G874R, L890Fand L957F.

All sequence data was also compared with the HCMV genome reference strain AD169 (GenBank accession No: BK000394) using SeqScape v2.5 software (Applied Biosystems, Foster City, USA).

Mutations were reported as resistance mutations, natural polymorphisms or unknown mutations. The predicted results were also compared with the list of mutations published in www.ViracorIBT.com (Viracol. IBT Laboratories) and with the Attachments I and II that report mutations described in the literature.

Chapter 4

Results

4.1. Characteristics of the study population

Our case series included 22 patients (9 female and 13 male), with a median age at allogeneic HSCT of 12 years (range: 0-60) – Table IV. The majority of the patients were submitted to allogeneic HSCT for acute leukemia (n=12, being *n* the frequency) with myeloblative conditioning regimen (n=15), and were transplanted with peripheral blood stem cells (n=11). All patients were seropositive for HCMV (positive for Immunoglobulin G (IgG) at the date of the transplant and received stem cells from both HCMV seropositive (n=10) or seronegative donors (n=12).

Table IV: Characteristics of the study population.

Variable	
Gender	**n**
Female	9
Male	13
Age, median (range) years old	12 (0-60)
Underlying disease	**n**
Acute leukemia	12
Chronic myeloproliferative diseases	1
Chronic lymphoproliferative diseases	2
Myelodysplatic/Myeloproliferative diseases	1
Aplastic anemia	3
Others	3
Stem cell source	**n**
Cord Blood	8
Bone Marrow	3
Peripheral Blood	11
Conditioning regimen	**n**
Reduced Intensity	7
Myeloblative	15
Donor	**n**
Match/Related	5
Mismatched or Unrelated	17
HCMV Donor/Recipient status	**n**
D-/R+	12
D+/R+	10

n, frequency; HCMV, Human cytomegalovirus; D, Donor; R, Recipient; D+, Positive Donor; R+, Positive Recipient; D-, Negative Donor; R-, Negative Recipient.

4.2. HCMV Infection

Table V summarizes the information regarding time to infection (TTI) and duration of infection (DOI) of HCMV, for each patient. Considering the TTI, 22 patients developed early HCMV infection (≤100 days post-transplant) with median TTI of 20.5 days (range 0-42) and median DOI of 108.5 days (range: 10-294).

Table V: Analysis of HCMV infection among allogeneic HSCT recipients.

Patient Number	Gender	Age (years old)	HCMV D/R status	TTI (days)	DOI (days)
1	Male	0	D-/R+	0	98
2	Male	24	D+/R+	28	39
3	Female	7	D-/R+	36	140
4	Male	1	D-/R+	16	187
5	Male	1	D-/R+	22	21
6	Male	10	D+/R+	39	31
7	Female	5	D+/R+	25	227
8	Male	2	D+/R+	42	53
9	Male	60	D+/R+	22	38
10	Female	49	D-/R+	18	273
11	Female	18	D-/R+	34	119
12	Male	49	D-/R+	18	168
13	Female	2	D-/R+	7	127
14	Female	2	D-/R+	19	196
15	Male	37	D+/R+	40	143
16	Male	41	D-/R+	7	84
17	Female	23	D+/R+	12	63
18	Male	1	D-/R+	17	14
19	Female	54	D+/R+	7	183
20	Male	30	D+/R+	32	10
21	Female	6	D+/R+	17	294
22	Male	14	D-/R+	28	70

HCMV, Human cytomegalovirus; D, Donor; R, Recipient; D+, Positive Donor; R+, Positive Recipient; D-, Negative Donor; R-, Negative Recipient; TTI, Time to Infection; DOI, Duration of Infection.

The evolution of HCMV infection with detailed viral information for each patient is shown in Attachment V.

4.3. Genotypic Analysis of *UL97* and *UL54* genes

4.3.1. *UL97* sequencing results

Table VI contains the summary of sequencing data (resistance mutations, polymorphisms and unknown mutations) of *UL97* for each patient by comparing the sequence information with Merlin and AD169 strains. Despite our efforts in the laboratory, there are 13/22 patients on which no complete sequence data was achieved, nevertheless, the missing data is outside the regions which have shown resistance mutations – Attachment I.

Overall, our data showed that five of the tweny two (23%) patients have been found with harbored HCMV resistance mutations (C592G, A594V, L595W and C603W) in *UL97*, being these mutations in bold in Table VI. The common polymorphisms, identified in our series, were: Q19E, N68D, S108N, L126Q, I244V, P247S and N510S. By comparing the sequence data with Merlin and AD169 strains, we have observed that there are a number of common unknown mutations found in our patients (S7Y, V96G, S135L, S155L, D167G, G188D, G242V, D269E, M330T, E333D, D422G, D430N, V466A, Y491H, R514H, V559A and A619S). We have found some unknown mutations that are strain specific (Merlin Strain: T75A, A104S, D122E, D290E and D422E), and indeed, all patients were mutant for T75A when compared with the Merlin strain. Only one unknown mutation was found in AD169 strain (E115K).

To summarize the frequency (n) of found mutations we have considered the reported mutations compared with AD169 strain: five known *UL97* resistance mutations, namely A594V (n=2), C592G (n=1), L595W (n=1) and C603W (n=1) were found; seven polymorphism, namely I244V (n=21), N68D (n=20), L126Q (n=10), Q19E (n=4), S108N (n=4), P247S (n=1) and N510S (n=1); and eighteen unknown mutations with the majority of the patients harboring more than one unknown mutation.

Table VI: Resistance mutations, polymorphisms and unknown mutations detected in *UL97* by Merlin and AD169 strains.

Patient Number	Merlin strain			AD169 strain		
	Resistance	**Polymorphism**	**Unknown**	**Resistance**	**Polymorphism**	**Unknown**
1	**L595W**	N510S	T75A	**L595W**	N68D, L126Q, I244V, N510S	-
2 [1)]	-	-	T75A	-	N68D, I244V	-
3 [2)]	-	Q126L	T75A	-	N68D, I244V	-
4 [3)]	**C592G**	-	T75A, G242V, D422G, A619S	**C592G**	N68D, L126Q, I244V	G242V, D422G, A619S
5	-	Q126L	T75A	-	N68D, I244V	-
6	-	-	T75A, V96G, E333D	-	N68D, L126Q, I244V	V96G, E333D
7	-	Q19E, S108N, Q126L	T75A	-	Q19E, N68D, S108N, I244V	-
8	-	-	T75A	-	N68D, I244V	-
9 [4)]	-	N510S	T75A, D122E, S135L, G188D, D269E	-	N68D, L126Q, I244V	E115K, S135L, G188D, D269E
10 [5)]	**A594V**	-	T75A, D422E, V466A, Y491H	**A594V**	N68D, L126Q, I244V	V466A, Y491H
11 [6)]	**A594V**	D68N, Q126L, V244I	T75A, S155L	**A594V**	-	S155L
12 [7)]	**C603W**	Q126L	T75A	**C603W**	N68D, I244V	-
13 [8)]	-	-	T75A, D167G, M330T, V559A	-	N68D, L126Q, I244V	D167G, M330T V559A
14	-	Q19E, S108N, Q126L	T75A	-	Q19E, N68D, S108N, I244V	-
15	-	-	T75A, D290E	-	N68D, L126Q, I244V	-
16 [9)]	-	-	T75A, R514H	-	N68D, L126Q, I244V	R514H
17 [10)]	-	Q126L, P247S	T75A	-	N68D, I244V, P247S	-
18	-	Q19E, S108N, Q126L	T75A	-	Q19E,N68D, S108N, I244V	-
19	-	-	T75A	-	N68D, L126Q, I244V	-
20 [11)]	-	-	S7Y, T75A, D430N	-	N68D, L126Q, I244V	S7Y, D430N
21 [12)]	-	D68N	T75A, A104S	-	I244V	-
22 [13)]	-	Q19E, S108N, Q126L	T75A	-	Q19E, N68D, S108N, I244V	-

1) Missing information of codon 149-151; **2) Missing** 149-152, 336-339; **3) Missing** 144-152, 336-339, 552-556; **4) Missing** 146-152; **5) Missing** 552-556; **6) Missing** 149; **7) Missing** 144-152; 336-339; 552-556; **8) Missing** 135-152; **9) Missing** 149-152, 336-339; **10) Missing** 149-152, 336-339; **11) Missing** 149-152, 336-339, 449-508; **12) Missing** 105-133, 152-166, 334-341; **13) Missing** 334-342, 548-558.

4.3.2. *UL54* sequencing results

Table VII contains the summary of sequencing data (resistance mutations, polymorphisms and unknown mutations) of *UL54*, for each patient by comparing the sequence information with TB40/E and AD169 strains. All patients were efficiently sequenced for *UL54*.

Of the studied patients, two out of twenty two (9%) harbored HCMV resistance mutations (P522S and L957F) in *UL54*, being these mutations in bold in Table VII. The common polymorphisms, identified in our series, were: A614S, S655L, F669L, S676G, N685S, A688V, G874R, A885T, ins885S, L890F, S897L and N898D. By comparing the sequence data with TB40/E and AD169 strains, we observed that there are only a few common unknown mutations found in our patients (S306I, S306T, L394F, Y477D and K493N). Moreover, there are two unknown mutations that are TB40/E strain specific (A559V and L845V) while only one was found in AD169 strain (S656L).

To summarize the frequency (n) of found mutations we have considered the reported mutations compared with AD169 strain: two resistance mutations P522S and L957F were found in one patient each; 12 polymorphisms, being A885T (n=18), N898D (n=14), S655L (n=9), N685S (n=9) and S897L (n=7) the most frequent; and 6 mutations of unknown significance (S306I, S306T, L394F, Y477D, K493N and S656L) were found with equal frequency (n=1).

Table VII: Resistance mutations, polymorphisms and unknown mutations detected in *UL54* by TB40/E and AD169 strains.

Patient Number	TB40/E strain			AD169 strain		
	Resistance	**Polymorphism**	**Unknown**	**Resistance**	**Polymorphism**	**Unknown**
1	-	L655S, S685N, S897L, D898N	-	-	-	-
2	-	L655S, S685N, S897L	-	-	S897L, N898D	-
3	-	A614S	L394F	-	A614S, S655L, N685S, A885T, N898D	L394F
4	-	F669L	-	-	S655L, F669L, N685S, A885T, N898D	-
5	-	ins885S, D898N	-	-	S655L, N685S, A885T, ins885S	-
6	**P522S**	-	-	**P522S**	S655L, N685S, A885T, N898D	-
7	-	L655S, S685N, T885A, D898N,	K493N	-	-	K493N
8	-	F669L	S306T, A559V	-	S655L, F669L, N685S, A885T, N898D	S306T
9	-	L665S, S685N, A688V, S897L	-	-	A688V, A885T, N898D	-
10	-	G874R, L890F	-	-	S655L, N685S, G874R, A885T, L890F, N898D	-
11	-	F669L	-	-	S655L, F669L, N685S, A885T, N898D	-
12	-	L655S, S685N, S897L	-	-	A885T, S897L, N898D	-
13	-	L655S, S685N, S897L, D898N	S306I, Y477D	-	A885T, S897L	S306I,Y477D
14	-	L655S, S676G, S685N, S897L	L845V	-	S676G, A885T, S897L, N898D	-
15	-	L655S, S685N, S897L, D898N	-	-	A885T, S897L	-
16	-	-	-	-	A885T, N898D	S656L
17	-	L655S, S685N, S897L	-	-	A885T, S897L, N898D	-
18	-	L655S, S685N, T885A, D898N	-	-	-	-
19	-	-	-	-	S655L, N685S, A885T, N898D	-
20	-	L655S, S685N, S897L, D898N	-	-	A885T, S897L	-
21	**L957F**	G874R, L890F	-	**L957F**	S655L, N685S, A885T, L890F, N898D	-
22	-	L655S, S685N, T885A, D898N	-	-	A885T	-

4.4. Characteristics of patients with resistance mutations

Table VIII contains the characteristics of the seven patients where resistance mutations were identified, being these mutations in bold. The first five patients had resistance mutations in *UL97* and the last two had resistance mutations in *UL54*. Additionally, this table also contains the polymorphisms and the unknown mutations that were found on each gene, for each patient, comparing with AD169 strain**.**

Among the seven out of the twenty two (32%) patients with resistance mutations, five of them contained resistance mutations in *UL97* (one patient each), and only two (one patient each) contained resistance mutations in the *UL54*. Patients with resistance mutations in *UL97* did not have resistance mutations detected in *UL54*. Within the five patients with resistance mutations in *UL97*, three are males and two are females; three patients were diagnosed with different underlying disease and two patients were diagnosed with acute myeloid leukemia; and all, except for one patient, were submitted to myeloblative conditioning regimen. The stem cell source was different: two received cord blood; two received peripheral blood and one received bone marrow. Curiously, all patients were transplanted with HCMV seronegative unrelated HLA-identical donor. Patients with resistance mutations in *UL54* did not have resistance mutations detected in *UL97*. The two patients with resistance mutations in *UL54* (one male and one female), were diagnosed with different underlying diseases: acute myeloid leukemia and Myelodysplatic/myeloproliferative disease, respectively. Both were submitted to myeloblative conditioning regimen, and received stem cells from peripheral blood (male) and from cord blood (female), both from an HCMV seropositive unrelated HLA-identical donor – Table VIII.

Regarding the outcome, all patients, except one which contains the *UL97* A594V resistance mutation, are dead – Table VIII.

Table VIII: Characteristics of patients with resistance mutations in the *UL97* and *UL54*.

Patient Number	Gender	Age (years/months old)	Underlying disease	Conditioning regimen	Stem cell source	HLA status	HCMV D/R status	Outcome	Detected mutations by AD169 strain	
									UL97	*UL54*
1	Male	< 12 months	Primary immunodeficiency	Myeloblative	Cord blood	Mismath or unrelated	D-/R+	Dead	**L595W,** N68D, L126Q, I244V, N510S	No mutations
4	Male	1	Acute lymphoid leukemia B linage	Myeloblative	Cord blood	Mismath or unrelated	D-/R+	Dead	**C592G,** N68D, L126Q, I244V,G242V, D422G, A619S	S655L, F669L, N685S, A885T, N898D
10	Female	49	Acute myeloid leukemia	Myeloblative	Peripheral blood	Mismath or unrelated	D-/R+	Dead	**A594V,** N68D, L126Q, I244V, V466A, Y491H	S655L, N685S, G874R, A885T, L890F, N898D
11	Female	18	Medullary aplasia	Reduced intensity	Bone marrow	Mismath or unrelated	D-/R+	Alive	**A594V,** S155L	S655L, F669L, N685S, A885T, N898D
12	Male	49	Acute myeloid leukemia	Myeloblative	Peripheral blood	Mismath or unrelated	D-/R+	Dead	**C603W,** N68D, I244V	A885T, S897L, N898D
6	Male	10	Acute myeloid leukemia	Myeloblative	Peripheral blood	Mismath or unrelated	D+/R+	Dead	N68D, L126Q, I244V, V96G, E333D	**P522S,** S655L, N685S, A885T, N898D
21	Female	6	Myelodysplatic/myeloproliferative disease	Myeloblative	Cord blood	Mismath or unrelated	D+/R+	Dead	I244V	**L957F,** S655L, N685S, A885T, L890F, N898D

Bold, Resistance Mutations; HLA, Human Leukocyte Antigen; HCMV, Human Cytomegalovirus; D, Donor; R, Recipient; D+, Positive Donor; R+, Positive Recipient; D-, Negative Donor; R-, Negative Recipient.

4.5. Analysis of HCMV infection and treatment in patients with resistance mutations

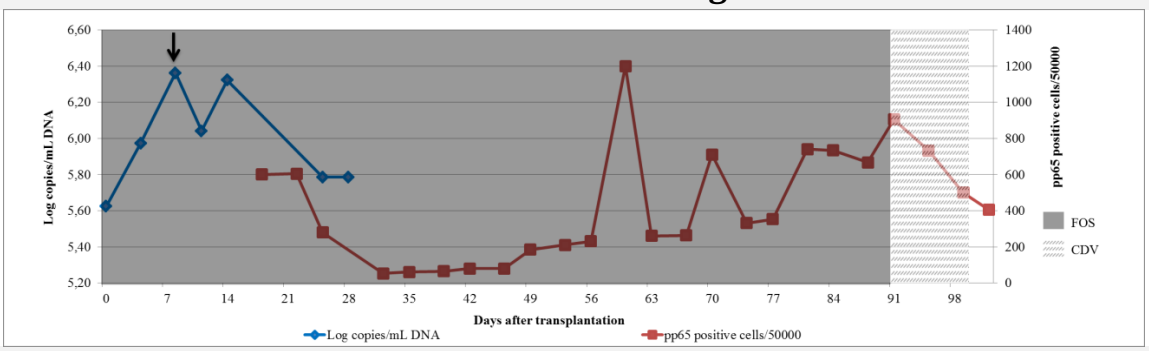
All patients included in this study were treated with preemptive treatment as described previously in the Material and Methods chapter. This subchapter describes each patient characteristics, HCMV monitoring and antiviral treatment (drug, dose and duration of the treatment) of the seven patients identified with resistance mutations in *UL97* and *UL54*. The first five patients (Patient 1, 4, 10, 11 and 12) had mutations in *UL97* and the last two (Patient 6 and 21) had resistance mutations in *UL54*.

The detailed information for all other patients is shown in Attachment V.

4.5.1. Patient 1

Patient 1 was a male with less than twelve months old, diagnosed with primary immunodeficiency submitted to myeloblative conditioning regimen, which received allogeneic HSCT cord blood from a HCMV seronegative unrelated HLA-identical donor.

HCMV monitoring



Antiviral Treatment

Day +0 – FOS 288 mg 8h/8h 35 days;
Day +41 – FOS 648 mg 1x/day 19 days;
Day +60 – FOS 432 mg 8h/8h 28 days;
Day +89 – FOS 648 mg 1x/day 2 day;
Day +91 – CDV 35 mg 1x/day 8 days.

Figure 14: HCMV monitoring and antiviral treatment of patient 1. The arrow points to the sample that was selected for mutations analysis.

The first episode of HCMV infection was detected on the day of the transplant (day +0). The treatment was initiated with FOS at day +0 until day +91. During this period of

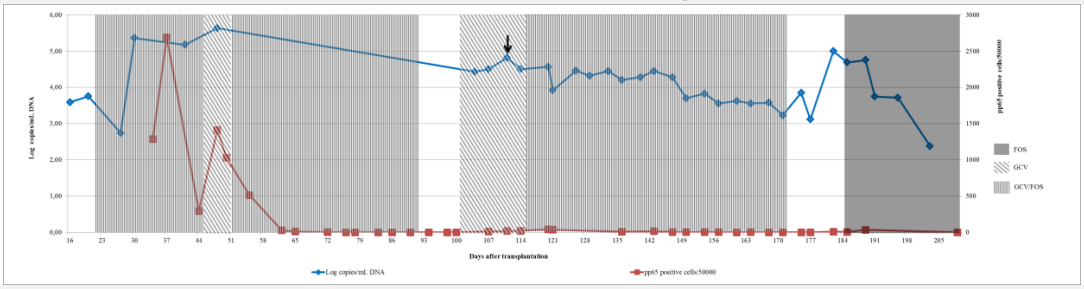
treatment, a decrease of viremia was observed after day +22, but was not achieved remission of infection, with an increase and persistence of viremia after day +60. Therapy was then switched to CDV, which was maintained during 8 days with the maintenance of infection – Figure 14.

In this patient, at day +8 after transplantation, we have identified the *UL97* L595W GCV/VGCV resistance mutation and four different polymorphisms in *UL97* (N68D, L126Q, I244V and N510S), but no mutations were found in *UL54* – Table VIII.

4.5.2. Patient 4

Patient 4, was a male with one year old diagnosed with acute Lymphoid Leukemia B lineage underwent myeloblative conditioning regimen, which received allogeneic HSCT cord blood from HCMV seronegative unrelated HLA-identical donor.

HCMV monitoring



Antiviral Treatment

Day +22 – GCV 44 mg 12h/12h 23 days;
Day +22 – FOS 504 mg 8h/8h 23 days;
Day +35 – GCV 43 mg 12h/12h 57 days;
Day +51 – FOS 520 mg 8h/8h 41 days;
Day +101 – GCV 40 mg 12h/12h 70 days;
Day +114 – FOS 480 mg 8h/8h 57 days;
Day +185 – FOS 508 mg 8h/8h 32 days.

Figure 15: HCMV monitoring and antiviral treatment of patient 4. The arrow points to the sample that was selected for mutations analysis.

The first episode of HCMV infection was detected in day +16 after the transplant. Preemptive therapy was initiated with the combination of GCV and FOS at day +22 until day +45. A slight decrease of the viremia was observed at the beginning of this treatment, but quickly increased until day +37, from when occurred a decrease of the viremia and the therapy was switched to GCV at day +45. At day +51, FOS was added to GCV until day +92 with a decrease of the number of positive cells and subsequent elimination in day +86.

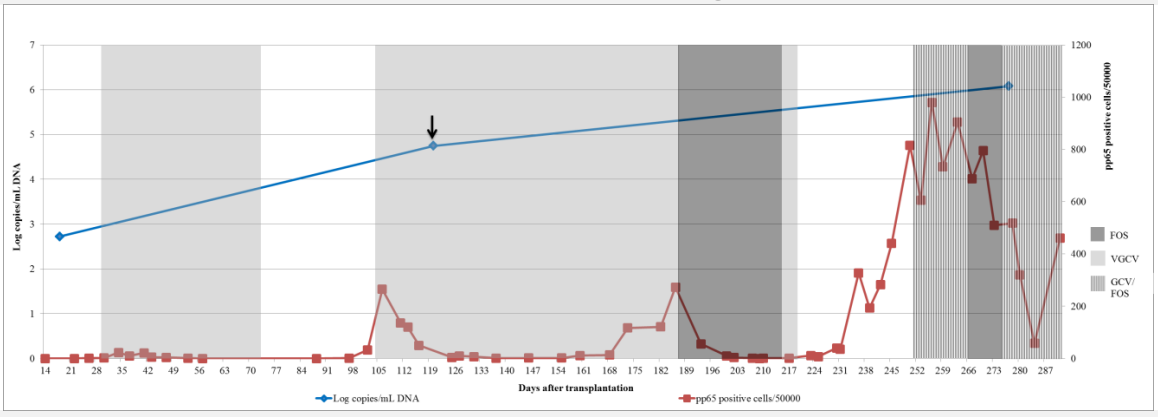
During the follow up, a reactivation occurred at day +94 and was treated with GCV (day +101 to day +114), which was replaced by therapy with GCV and FOS (day +114 to day +171). During the period of the treatment with GCV, the viremia increased, but with the administration of the combination of GCV and FOS therapy, the viral load/positive cells slightly decreased. The viral load/positive cells increased again, but decreased after the administration of FOS therapy from day +185 with subsequent elimination of HCMV (0 positive cells) at day +209 – Figure 15.

At day +111 after transplantation, we identified, in *UL97*, the C592G GCV/VGCV resistance mutation, three different polymorphism (N68D, L126Q and I244V) and three unknown mutations (G242V, D422G and A619S); while in *UL54* five different polymorphism were found (S655L, F669L, N685S, A885T and N898D) – Table VIII.

4.5.3. Patient 10

Patient 10 was a 49 years old female with acute myeloid leukemia submitted to myeloblative conditioning regimen, which received allogeneic HSCT peripheral blood from HCMV seronegative unrelated HLA-identical donor.

HCMV monitoring



Antiviral Treatment

Day +30 – VGCV 900 mg 12h/12h 44 days;	**Day +211** – VGCV 450 mg 12h/12h 6 days;
Day +103 – VGCV 900 mg 12h/12h 13 days;	**Day +251** – GCV 250 mg 12h/12h 16 days;
Day +120 – VGCV 900 mg 12h/12h 5 days;	**Day +252** – FOS 3288 mg 8h/8h 22 days;
Day +131 – VGCV 900 mg 12h/12h 55 days;	**Day +276** – GCV 280 mg 12h/12h 15 days;
Day +186 – FOS 3288 mg 8h/8h 25 days;	**Day +276** – FOS 3360 mg 8h/8h 15 days.

Figure 16: HCMV monitoring and antiviral treatment of patient 10. The arrow points to the sample that was selected for mutations analysis.

The first episode of HCMV infection was detected in day +18 after the transplant, and the preemptive treatment was initiated with VGCV (day +30 to day +74). At the beginning of the treatment with VGCV it was observed an increase of the number of positive cells until day +41, from this day forward was observed a decrease, and subsequent elimination, at day +57 (0 positive cells). At day +97, it was detected a new episode of infection, and the therapy was initiated with VGCV from day +103 until day +186. At day +186, the therapy was switched to FOS and the viremia decreased until elimination at day +209. A reactivation was identified at day +210 and the number of positive cells increased but with the administration of combination therapy, GCV and FOS, the number of positive cells slightly decreased. The continuous therapy with FOS leads to a decrease of the number of positive cells. The therapy was again switched to a combination of GCV and FOS, which was maintained until the patient died – Figure 16.

In this patient, at day +120 after transplantation, we have identified the A594V GCV/VGCV resistance mutation, three different polymorphism (N68D, L126Q and I244V) and two unknown mutations (V466A and Y491H) in *UL97*; and six different polymorphism in *UL54* (S655L, N685S, G874R, A885T, L890F and N898D) – Table VIII.

4.5.4. Patient 11

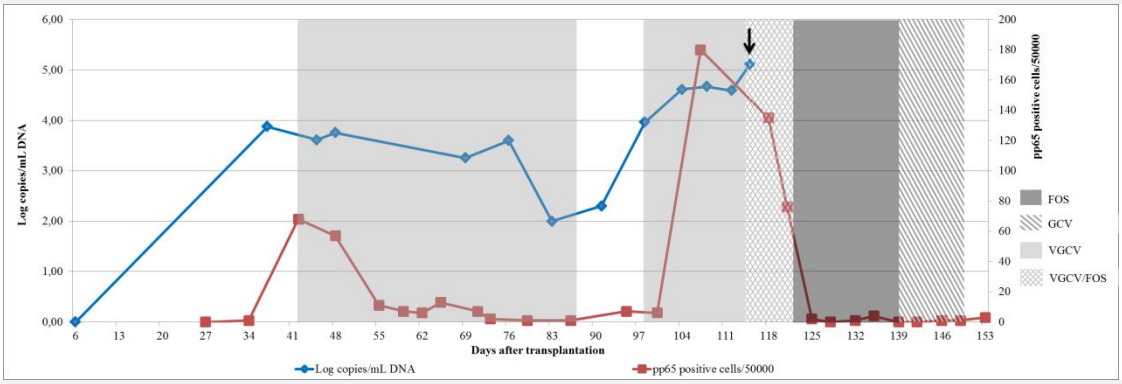
Patient 11 was an 18 years old female with a medullary aplasia underwent a reduced intensity regimen, which received allogeneic HSCT bone marrow from HCMV seronegative unrelated HLA-identical donor.

The first episode of HCMV infection was detected in day +34 after the transplant. Preemptive treatment was initiated with VGCV, from day +42 until day +86. No significant change in viremia was observed under VGCV therapy. Indeed, after inclusion of FOS (day +115) in combination with VGCV, followed by FOS therapy alone there was a significant decrease of HCMV viremia and subsequent elimination, at day +139. The therapy was then switched to GCV (day +139 to day +149), which kept the number of positive cells in a low level – Figure 17.

In this patient, we have identified, at day +115 after transplant, the A594V GCV/VGCV resistance mutation and one unknown mutation, S155L, in *UL97*, and five

different polymorphisms, S655L, F669L, N685S, A885T and N898D, in *UL54* – Table VIII.

HCMV monitoring



Antiviral Treatment

Day +42 – VGCV 900 mg 12h/12h 41 days;
Day +84 – VGCV 450 mg 12h/12h 2 days;
Day +98 – VGCV 900 mg 12h/12h 24 days;
Day +115 – FOS 2640 mg 12h/12h 3 days;
Day +119 – FOS 4080 mg 8h/8h 2 days;
Day +122 – FOS 3360 mg 12h/12h 4 days;
Day +123 – FOS 4080 mg 1x/day 8 days;
Day +132 – FOS 2688 mg 12h/12h 1 day;
Day +133 – FOS 4080 mg 1x/day 6 days;
Day +139 – GCV 300 mg 1x/day 2 days;
Day +142 – GCV 170 mg 1x/day 7 days.

Figure 17: HCMV monitoring and antiviral treatment of patient 11. The arrow points to the sample that was selected for mutations analysis.

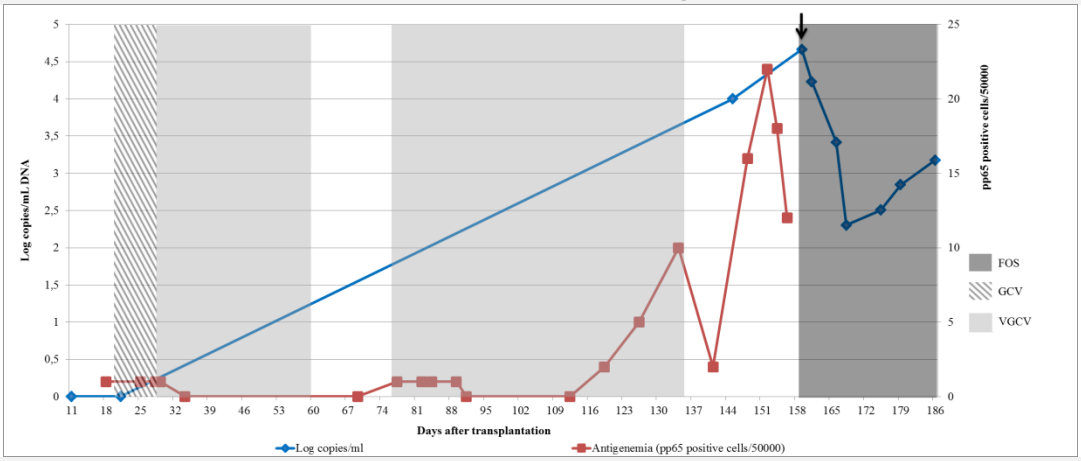
4.5.5. Patient 12

Patient 12 was a 49 years old male with an acute myeloid leukemia submitted to myeloblative conditioning regimen, which received allogeneic HSCT peripheral blood from HCMV seronegative unrelated HLA-identical donor

The first episode of HCMV infection was detected in day +18 after the transplant and was treated with GCV therapy, from day +19 until day +25 and changed to VGCV under maintenance therapy. At day +119, a new episode of infection started, with HCMV viremia increasing significantly under VGCV therapy and remaining high until administration of FOS at day +159. Afterwards there was a slightly decreased in viremia without elimination under FOS therapy – Figure 18.

In this patient, it was observed, at day +159 after transplant, the *UL97* C603W GCV/VGCV resistance mutation, two different polymorphisms (N68D and I244V) in the *UL97*, and three different polymorphisms, A885T, S897L and N898D, in *UL54* – Table VIII.

HCMV monitoring



Antiviral Treatment

Day +19 – GCV 450 mg 12h/12h 6 days; **Day +28** – VGCV 900 mg 2 days; **Day +39** – VGCV 900 mg 4 days; **Day +54** – VGCV 450 mg 6 days;	**Day +77** – VGCV 900 mg 57 days; **Day +159** – FOS 3600 mg 8h/8h 20 days; **Day +172** – FOS 3120 mg 12h/12h 15 days.

Figure 18: HCMV monitoring and antiviral treatment of patient 12. The arrow points to the sample that was selected for mutations analysis.

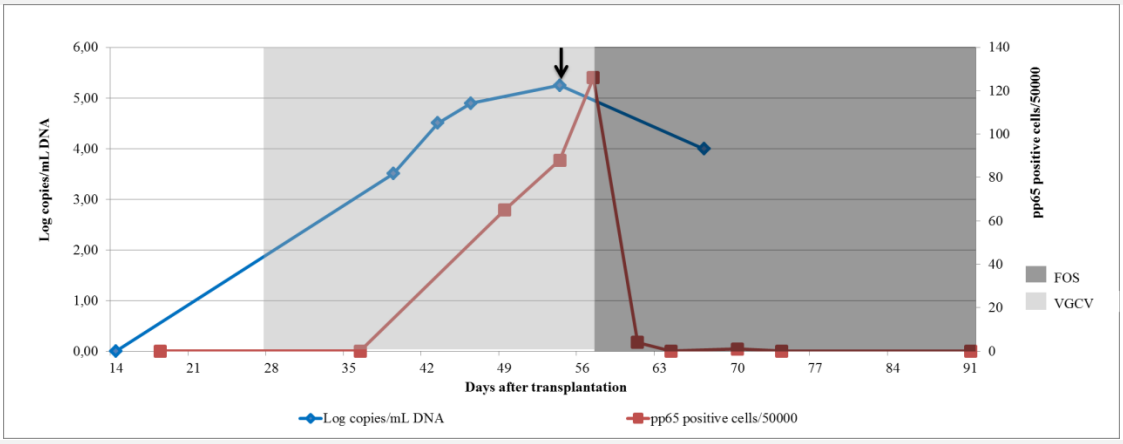
4.5.6. Patient 6

Patient 6 was a 10 years old male with an acute myeloid leukemia submitted to myeloblative conditioning regimen, which received allogeneic HSCT peripheral blood from HCMV seropositive unrelated HLA-identical donor.

The first episode of HCMV infection was detected in day +39 after the transplant, when the patient was already being administered with VGCV therapy. The viremia increased significantly, under VGCV therapy, until day +57, when FOS was administered. During the therapy with FOS it was observed a decrease of the viral load/positive cells and subsequent elimination, at day +64 – Figure 19.

In this patient, it was observed, at day +54 after transplant, the *UL54* P522S mutation, known to be associated with a cross-resistance to GCV/VGCV and CDV, four different polymorphisms S655L, N685S, A885T and N898D, in *UL54* and three different polymorphisms, N68D, L126Q and I244V, and two unknown mutations, V96G and E333D in the *UL97* – Table VIII.

HCMV monitoring



Antiviral Treatment

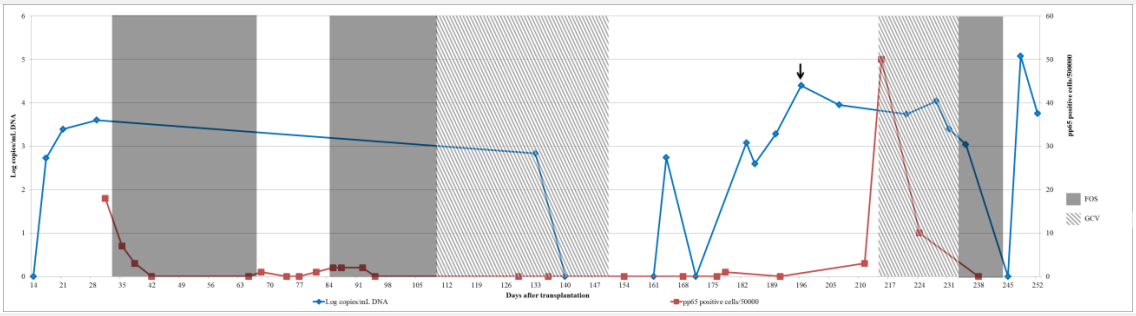
Day +28 – VGCV 450 mg 12h/12h 29 days;	**Day +75** – FOS 2808 mg 1x/day 16 days;
Day +57 – FOS 1848 mg 8h/8h 32 days;	**Day +91** – FOS 1224 mg 8h/8h.

Figure 19: HCMV monitoring and antiviral treatment of patient 6. The arrow points to the sample that was selected for mutations analysis.

4.5.7. Patient 21

Patient 21 was a 6 years old female with a myelodysplatic/myeloproliferative disease underwent myeloblative conditioning regimen, which received allogeneic HSCT cord blood from HCMV seropositive unrelated HLA-identical donor.

HCMV monitoring



Antiviral Treatment

Day +32 – FOS 1080 mg 8h/8h 17 days;	**Day +133** –GCV 50 mg 1x/day 16 days;
Day +50 – FOS 1344 mg 8h/8h 16 days;	**Day +215** – GCV 52.5 mg 12h/12h 6 days;
Day +85 – FOS 1121 mg 8h/8h 24 days;	**Day +221** – GCV 16 mg 1x/day 7 days;
Day +109 – GCV 100 mg 12h/12h 4 days;	**Day +228** – GCV 50 mg 1x/day 4 days;
Day +114 – GCV 50 mg 12h/12h 19 days;	**Day +232** – FOS 220 mg 8h/8h 9 days.

Figure 20: HCMV monitoring and antiviral treatment of patient 21. The arrow points to the sample that was selected for mutations analysis.

The first episode of HCMV infection was detected in day +17, after the transplant. The preemptive treatment was initiated at day +32 with FOS, which was extended until day +66. During the FOS therapy, the number of positive cells decreased leading to its subsequent elimination (0 positive cells) at day +42. A new HCMV episode occurred and was treated with FOS, during day +85 until day +109. Therapy was then switched to GCV, from day +109 until day +149, during when, the infection remained absent, with only one positive result for HCMV by quantitative PCR, at day +133. During the interval when no therapy was administered, from day +154 until day +211, the viremia was inconstant, with some episodes of positivity by quantitative PCR. At day +215, GCV therapy was initiated until day +232 with a slightly decreased of HCMV infection. Afterwards, the therapy was changed to FOS and the infection was eliminated at day +238 for a short period – Figure 20.

In this patient, it was observed, at day +196 after transplant, the *UL54* L957F mutation, known to be associated with a resistance to GCV/VGCV, five different polymorphisms, S655L, N685S, A885T, L890F and N898D, in *UL54* and one polymorphism, I244V, in *UL97* – Table VIII.

Chapter 5

Discussion

The development of HCMV drug resistance in the transplanted population appears to vary widely depending on the type of patients, and remains relatively rare for hematopoietic stem cell transplanted (HSCT) patients [74, 79, 142]. Indeed, there are some retrospective and prospective studies, to analyze HCMV drug-resistant mutations on allogeneic HSCT (allo-HSCT) patients worldwide but not from Portugal [73, 93, 103, 142, 145, 167]. In this study, we have retrospectively analyzed a population of patients submitted to allo-HSCT with diagnosed HCMV infection on which we have attempted to identify drug-resistant mutations.

5.1. Correlation of HCMV resistance mutations with antiviral treatment response

In the population in study we have identified seven patients with resistance mutations. Of the seven patients, the first five (patient 1, 4, 10, 11 and 12) had resistance mutations in *UL97* and the last two (patient 6 and 21) had resistance mutations in *UL54*. The correlation of HCMV resistance mutations with the antiviral treatment response, for each one of these patients, will be discussed in the following lines.

5.1.1. Patient 1

This patient was diagnosed with a HCMV infection at the day of transplant (day +0) being treated with FOS. At day +8 we identified the *UL97* L595W mutation, which is known to confer a moderate GCV/VGCV resistance [108, 123]. As this mutation confers resistance to GCV/VGCV it was expected a decrease and subsequent elimination of the infection, however, the remission of the infection was not achieved. Besides the

identification of four *UL97* polymorphisms (N68D, L126Q, I244V and N510S) we have not identified any mutation in *UL54.* Considering that patient's response to FOS was not complete, we hypothesize that other HCMV strains may coexist, which may contain FOS resistance mutations. Indeed, it has been reported that the continuous administration of a drug can lead to the accumulation of multiple drug resistance mutations, which may explain the increase and persistence of viremia after day +60 [74, 96, 99, 100]. Therapy was then switch to CDV, which seemed to be effective, although the patient has died. The use of CDV as a second-line therapy is indicated especially to rescue patients failing antiviral therapy with GCV, FOS or both [62, 90].

5.1.2. Patient 4

In this patient, HCMV infection was detected at day +16 with very high HCMV levels and the treatment was initiated at day +22 with the combination of GCV and FOS, only after two consecutive positive results. Despite it has been reported that high viral load/positive cells at the start of the therapy may contribute to the emergence of resistance, the combination of GCV and FOS has been used with high effectiveness in patients with a high viral load [19, 73, 90, 91]. Indeed, there was a decrease of viremia and its subsequent elimination in day +86. Nevertheless, a reactivation occurred immediately at day +94, with an increase in the viremia. The treatment was then initiated with GCV and afterward combined with FOS at day +114. At day +111 we identified the *UL97* C592G mutation, which is reported to confer a low level of resistance to GCV/VGCV and preferentially selected in situations where the virus is exposed to low GCV concentrations [55, 112]. In addition, this mutation may have emerged from prolonged antiviral therapy. Even though this mutation confer low resistance, it is likely to be responsible for the viral persistence. Hence, the decrease and subsequent elimination of HCMV viremia, at day +209 is likely to be conferred by FOS. We have also found polymorphisms in *UL97* (N68D, L126Q and I244V) and *UL54* (S655L, F669L, N685S, A885T and N898D), and some unknown mutations in *UL97* (G242V, D422G and A619S) that may play an important role in the emergence of antiviral resistance.

5.1.3. Patient 10

In this patient, the infection was detected at day +18 and the treatment initiated with VGCV at day +30, leading to the elimination of the HCMV infection episode at day +57. However, a new HCMV episode started at day +97 and despite the therapy with VGCV, the infection was not eliminated. Indeed, we have detected the *UL97* A594V mutation at day +120. This mutation is known for being frequent and for confer high resistance to GCV/VGCV [112, 137, 153]. Additionally, several polymorphisms were found in *UL97* (N68D, L126Q and I244V) and in *UL54* (S655L, N685S, G874R, A885T, L890F and N898D).Two unknown mutations in *UL97* (V466A and Y491H) were also observed. Curiously, despite VGCV resistance, the number of positive cells remained relatively low under VGCV until day +155. However, this mutation is likely to be responsible for the viral persistence and for the increase of the number of positive cells, which occur at day +155 until day +186. In this day, the treatment was changed to FOS and lead to undetected levels of HCMV until day +209. Nevertheless, a reactivation occurred at day +210, which persisted for a long period even with a combination therapy of GCV and FOS. This persistence may be due to possible GCV and FOS resistance developed during the long period of treatment [99].

5.1.4. Patient 11

Patient 11 was detected with HCMV infection at day +34 and treated with VGCV, which was responsible for the decrease of infection until day +83. Nevertheless, the HCMV levels increased by day +91 and the therapeutics were combined with FOS at day +115. In this day we have identified the *UL97* A594V mutation, known to confer high resistance to GCV/VGCV [112, 137, 153]. This resistant strain, which may have emerged from a prolonged therapy with VGCV, could have been the cause of the increase and persistence of the viral replication. Additionally, several polymorphisms (S655L, F669L, N685S, A885T and N898D) were found in *UL54*, and one unknown mutation (S155L) in *UL97*. After the combination of FOS with VGCV, followed by FOS therapy alone, there was a significant decreased of HCMV viremia and subsequent elimination at day +139, probably due to the effect of FOS. Afterwards, the therapeutics changed to GCV and the

infection was maintained under low levels, which may be the result of the emergence of GCV resistance strains.

5.1.5. Patient 12

This patient was positive for HCMV at day +18, whom had the therapy initiated with GCV and then changed to VGCV with elimination of the infection by day +34. A new HCMV episode started at day +77 and was eliminated at day +91, with VGCV therapy. Nevertheless, under VGCV therapy, a new infection occurred at day +119 with increasing HCMV viremia and the therapeutics was changed, at day +159, to FOS. In this day, we detected the *UL97* C603W mutation, which is known to be associated with a high GCV/VGCV resistance [101, 153] and might be the result of prolonged therapy with VGCV and the responsible for the increase of HCMV viremia under VGCV therapy. We have also found the following polymorphisms: N68D and I244V in *UL97*, and A885T, S897L and N898D in *UL54*, which may modulate the resistance level conferred by this mutation [100, 136]. However, with the introduction of the FOS therapy the viremia has decreased, but without remission of the infection, and so other strains with FOS resistant mutations may co-exist.

5.1.6. Patient 6

In this patient, the HCMV infection was detected at day +39 after the transplant, when the patient was already being administered with VGCV. The viremia increased, under the therapy with VGCV, until day +57. This increase is likely to have resulted from the resistant strain that emerged at day +54, with a *UL54* P522S mutation, which is known for confer dual low-grade GCV-CDV resistance [74, 156]. Additionally, we have detected polymorphisms in *UL54* (S655L, N685S, A885T and N898D) and in *UL97* (N68D, L126Q and I244V), which may modulate the drug-resistance level provided by resistance mutation [100, 136], and two unknown mutations (V96G and E333D) in *UL97*, which may be of potential interest. Hence, with the switch of therapy to FOS at day +57, we observed a significant reduction and subsequent elimination of the HCMV infection by day +64.

5.1.7. Patient 21

In this patient, the infection was detected at day +17, but the therapy was only initiated at day +32 with FOS, when HCMV viremia was already high. It has been reported that high viral load/positive cells at the start of the therapy may contribute to the emergence of resistance [19, 73]. However, despite the therapy started while the viremia was high, the infection was eliminated with FOS at day +42. New HCMV infection episodes and reactivations occurred from day +68 until day +191 that were eliminated by the treatment or possibility by the host immune response of the individual. During the interval when no therapy was administered, a reactivation occured at day +196, when we have identified the *UL54* L957F mutation, which is known to confer low resistance to GCV/VGCV [98, 105]. Additionally, the following polymorphisms were found: S655L, N685S, A885T and N898D in *UL54* and I244V in *UL97*. Under the therapy with GCV, despite occuring a slight reduction of the HCMV infection, which may be due to the fact that this mutation confers low resistance to GCV, this mutation is likely to be responsible for the viral persistence, that ends up by being eliminated with the switch of the therapy to FOS.

5.2. Overall Discussion

This is the first study in Portugal to characterize both HCMV *UL97* and *UL54* sequences and to identify HCMV drug-resistance mutations in allogeneic HSCT patients. The majority of the studies use AD169 reference strain, to analyze mutations in both genes. In our study, in addition to the AD169 strain, we also used the Merlin and TB40/E strains, which were used to analyze mutations on genes *UL97* and *UL54*, respectively. Even though we had our focus on the results obtained by the AD169 strain, there were identified mutations that are specific to Merlin or TB40/E strains, which may be relevant for the emergence of HCMV resistance.

Our study was developed with twenty two patients on whom we have been able to identify seven (32%) with resistance mutations: five (23%) with resistance mutations in the *UL97* (one patient each); and two (9%) with resistance mutations in *UL54* (one patient each). In immunocompromised patients, such as HSCT, the treatment of HCMV

infection/disease often require prolonged administration of antiviral therapy [96] and it has been shown that prolonged treatment (e.g. several months) can promote the development of resistant HCMV strains resulting in an impaired response to the therapy [98, 99]. In our study, some drug-resistant HCMV mutations were first detected 2-3 months after antiviral therapy. It is known that the occurrence of drug resistance during the first six weeks of therapy is unusual, although this has been reported to occur [129, 168]. In our study, some drug-resistant HCMV mutations were first detected during the first six weeks of therapy. Moreover, the continuous administration of a drug to which resistance was observed in some patients can lead to accumulation of multiple drug resistance mutations [74, 99, 100]. Additionally, it has also been reported that suboptimal antiviral concentrations due to poor compliance or low drug absorption and limited oral bioavailability are others risk factors in the emergence of HCMV drug resistance [45, 68, 73, 96].

Ganciclovir/Valganciclovir (GCV/VGCV) resistance in HCMV is usually caused by mutations in the *UL97* kinase gene, which impair the initial phosphorylation of the drug that is necessary for its antiviral activity [58, 62]. The GCV/VGCV resistance mutations in *UL97* that were found in this study were C592G, A594V, L595W and C603W. By revising the literature, these amino acid changes belong to the most frequently detected mutations with a known confirmed GCV/VGCV resistant [108-110, 131]. Additionally, these mutations are clustered in the codon segment 590–607 (non-functional region), which is dispensable for viral replication [19, 40, 55, 77, 81]. Nevertheless, a considerable diversity of mutations, in this region, has been linked to varying degrees of GCV/VGCV resistance, which impair the recognition of GCV as a substrate while preserving the normal biological function [19, 55]. Of the four resistance mutations identified in our patients, the A594V mutation was the most frequent and has been frequently found in several other studies [116, 137, 153], being the one conferring more resistance to GCV/VGCV ($IC_{50=}$8.3) [101, 112]. Except for C592G, which confers low-grade resistance (IC_{50}=2.9), the L595W (IC_{50}=5.1) and C603W (IC_{50}=8.0) confer moderate to high-grade resistance [55, 101, 112]. Additionally, we observed that patients with GCV/VGCV-resistant *UL97* mutations had no *UL54* resistance mutations, which is in accordance with the literature since the major contribution for GCV/VGCV resistance is on *UL97* [73, 99, 116, 153].

In contrast with *UL97*, *UL54* resistance mutations are less common and can confer resistance to any, or all, of the current HCMV antivirals (GCV, VGCV, FOS and CDV) [75, 76, 97]. The *UL54* P522S mutation, located on exonuclease domains (N-terminal

extremity of ∂-region C), was observed in one patient and has shown to confer low-grade resistance to GCV/VGCV (IC_{50}=3.1) and to CDV (IC_{50}=3.6) [40, 77, 98, 140, 156]. This mutation is located in a region of frequent mutations that confer resistance to GCV/VGCV and CDV and is among the most frequent mutations, in *UL54*, associated with drug resistance [77, 99]. The other *UL54* mutation found in our study was the L957F, which is known to confer low resistance to GCV/VGCV (IC_{50}=2.7) [98, 105]. This mutation is one of the few *UL54* mutations that confer resistance only to GCV/VGCV and, until now, was only reported on laboratory strains [98, 105] and despite the fact that the majority of the resistance mutations in *UL54* occur within the conserved regions, this mutation occurs outside of these regions [19]. Our study showed that patients with resistance mutations in *UL54* did not have resistance mutations in *UL97,* and although uncommon this has been reported previously [138, 139]. Nevertheless, we have found, in one patient with *UL54* resistance mutation, that there were *UL97* mutations of unknown significance, which may be of potential interest.

The level of resistance is a very important factor that influences patient's outcome [93, 147]. In our study, we observed that of the seven patients with resistant mutations, only one is alive, and he had a high resistance mutation. Indeed, patients with mutations that conferred low-resistance had the same outcome (death) as the others. The only patient alive was submitted to a bone marrow transplant with reduced intensity regimen. Thus, in our study, the immunosuppression regimens and the source of stem cells may have influenced the clinical outcome. Moreover, most HCMV drug resistance clinical reports have shown the more severe outcomes, because the same host factors that predispose to drug resistance are also associated with more severe HCMV disease [19]. A recent study showed that HCMV drug resistance mutations were not associated with a poor outcome [73]. This leads us to think that other factors other than the level of resistance of the mutations may be important to define the clinical outcome.

UL97 and *UL54* variants, known in literature as natural polymorphisms, were also observed in seven patients with resistance mutations. The *UL97* observed variants were: N68D, L126Q, I244V and N510S, being I244V (n=6), N68D (n=5) and L126Q (n=4) the most frequent. Except for the N510S polymorphism, all others occur outside the region where *UL97* polymorphisms are usually found (on codon range 460-607) [136] and all of these polymorphisms were also found in other studies [55, 93, 101, 136, 169]. The *UL54*

observed variants were: S655L, F669L, N685S, G874R, A885T, L890F, S897L and N898D. These variants occur at residues outside the conserved regions, such as the majority of the polymorphisms found in this gene, and all of them were also found in other studies [19, 78]. The A885T (n=6), N898D (n=6), S655L (n=5) and N685S (n=5) variants were the most frequently found, such as observed in others studies [73, 82, 93, 94, 116, 146, 156]. It has been reported that some natural polymorphisms may modulate the drug-resistance level provided by other mutations [100, 136]. In our study, these polymorphisms, identified in *UL97* or in *UL54*, were found in combination with *UL97* and *UL54* resistance mutations. Other recent studies also showed that the polymorphisms identified in the *UL97* or in the *UL54* are found in combination with resistance mutations [73, 82, 93, 94, 116]. For example, the N510S polymorphism, which was found in just one patient, was found in a HCMV isolate resistant to GCV with a deletion of *UL97* codons 591-594 [108]. In our study, this polymorphism was detected in a HCMV isolate resistant to GCV, with a L595W mutation. Thus, the role of these mutations, found in *UL97* and *UL54*, as polymorphism or compensatory mutation remains unclear and needs further investigation.

Moreover, eight new mutations of unknown significance (V96G, S155L, G242V, E333D, D422G, V466A, Y491H and A619S) found in *UL97* were observed in three patients with *UL97* resistance mutations and in one patient with *UL54* resistance mutation. The D422G and A619S unknown mutations, found in the patient with the *UL97* C592G mutation, and V466A and Y491H found in one patient with the *UL97* A594V mutation, are located between different conserved regions of the protein and are clustered in the codons segment (342-708) where all known GCV/VGCV-resistance mutations have been described [97, 107]. In addition, the majority of the *UL97* resistance mutations are found outside the conserved kinase domains, which are more likely to be critical to the overall kinase function [19, 40, 55, 77, 108-110]. Some amino acid positions of *UL97* can be involved in both natural polymorphisms and antiviral resistance [136]. Additionally, the V466A unknown mutation occurs at the same position as a previous established GCV/VGCV resistance-associated substitution (V466G) and natural polymorphism (V466M) and, therefore, its role needs further investigation. The V96G and E333D found in patient with *UL54* P522S mutation, the G242V found in the patient with the *UL97* C592G mutation and S155L found in one patient with the *UL97* A594V mutation, occur at a region of the gene where, until now, resistance mutations to GCV/VGCV were not

found. However, a recent study found the F342S mutation in the region I of the gene, which confers a moderate resistance to GCV/VGCV, where, until then, resistance mutations to GCV/VGCV had not yet been described in these regions [117]. In addition, studies describe the *UL97* natural polymorphisms mainly focused on codon range 460-607, where all known GCV/VGCV resistance mutations are localized [55]. Nevertheless, phenotypic studies are required to ascertain the true nature of these new mutations.

The clinical courses of HCMV infection are influenced by multiple risk factors as well as by the presence of an antiviral-resistance mutation [93]. Our patients met some critical risk factors for the emergence of HCMV resistance: the majority were high-risk patients for HCMV infection with HCMV D-/R+ serostatus; some received stem cells from cord blood; and, except for one patient, all were submitted to myeloblative conditioning regimen [40, 73, 93]. High viral load/positive cells at the start of the therapy and recurrent HCMV infections were other risk factors, which may have contributed to the emergence of resistance in some of these patients [19, 77, 93]. The patients where *UL97* resistance mutations were found were transplanted with HCMV seronegative unrelated HLA-identical donor while patients with *UL54* resistance mutations were transplanted with HCMV seropositive unrelated HLA-identical donors. Curiously, *UL97* resistance mutations that confer low to moderate resistance to GCV/VGCV appeared on the patients that were transplanted with cord blood while the ones conferring high resistance appeared on the patients transplanted with peripheral blood or bone marrow. In *UL54*, the L957F mutation, which confers low resistance to GCV, appeared on the patient transplanted with cord blood while the P522S mutation appeared on the patient transplanted with peripheral blood. This observation leads us to presume that different factors may have contributed to the emergence of different resistance mutations.

In the patients where resistance mutations were not found, we have found unknown mutations in both *UL97* and *UL54*, which may have influenced the development of resistance. In *UL97*, the majority of mutations occur at a region of the gene where, until now, resistance mutations to GCV/VGCV were not found: S7Y, E115K, S135L, D167G, G188D, D269E and M330T. Nevertheless, D430N, R514H and V559A mutations, are located between different conserved regions of the protein and all them are clustered in the codons segment (342-708) where all known GCV/VGCV-resistance mutations have been described and where *UL97* polymorphisms are usually found [55, 97, 107]. In *UL54*, the

L394F, K493N and S306T/I mutations are located within the exonuclease domains where other resistance mutations to GCV/VGCV and CDV are more frequent [40, 77, 98], including the mutation found in this study (P522S). Because of their location inside the conserved domains, all of these mutations are likely considered to be associated to antiviral resistance, rather than to polymorphisms. Additionally, *UL54* Y477D and S656L mutations, due to their location outside the conserved regions, are likely considered to be associated with polymorphism, rather than to resistance mutations. Despite this assumption, more studies are required to ascertain the true nature of these new mutations.

Chapter 6

Conclusions and Future Directions

The emergence of HCMV resistance to one or more antiviral agents, which is influenced by multiple risk factors as well as by the presence of antiviral-resistance mutations, is associated with treatment failure and progression of HCMV disease in immunocompromised patients, complicating therapeutic and clinical management. In our study we have found HCMV drug-resistance mutations, in both *UL97* and *UL54*, leading to an impaired response to the therapy.

Our results confirm predominant role of *UL97* mutations in HCMV resistance to GCV/VGCV, which are in accordance with previous studies [73, 94, 99]. In this gene, we have detected the C592G, A594V, L595W and C603W mutations, which belong to the most frequently detected mutations. *UL54* gene was less frequently mutated than *UL97*, and the detected mutations were the P522S and L957F in the absence of mutations in *UL97*. *UL54* P522S mutation is located in a region of frequent mutations that confer resistance to GCV/VGCV and CDV and is among the most frequent *UL54* mutations associated with drug resistance. Furthermore, to our knowledge, this was the first study describing the L957F mutation among immunocompromised patients. Until now, this mutation was only reported on laboratory strains. These mutations confer different resistance levels and, based on the clinicopathological data for each patient, we conclude that different factors may have contributed to the emergence of mutations with different levels of resistance. Moreover, we also conclude that other factors other than the level of resistance of the mutations may be important to define the clinical outcome. Additionally, several polymorphisms were found in both genes, in combination with *UL97* and *UL54* resistance mutations, which may modulate the drug-resistance level provided by resistance mutations. Unknown mutations in *UL97* and/or in *UL54* were also found and they may play an important role in the emergence of antiviral resistance. However, further investigation is required to understand the role of these mutations as polymorphisms or

compensatory mutations and phenotypic studies are required to ascertain the true nature of the unknown mutations.

Given the frequency of resistance mutations found in this study (n=7, 32%), we conclude that it is important to implement protocols for a rapid detection of HCMV mutations in cases of clinical and laboratory suspicion, improving the management of patients. The establishment of guidelines for HCMV treatment, including genotyping information of viral genes, is important to improve the management of allogeneic HSCT patients with alternative treatments.

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Attachments

Attachment I: Mutations in viral phosphokinase (*UL97*) gene associated with GCV/VGCV resistance in laboratory and clinical isolates confirmed by marked transfer.

Mutation	Amino acid change		Viral Phenotype [a] GCV/VGCV IC_{50}	References
	Wild type (Wt)	Mutant		
F342S	F	S	R (7.8)	[117]
del355	K	del 1	R (16)	[117]
V356G	V	G	R (5.5)	[117]
L405P	L	P	R (2.5)	[101]
D456N	D	N	R (12)	[118]
M460I	**M**	**I**	**R (5)**	**[73, 96, 104, 108, 116, 134, 144, 157, 159, 170, 171]**
M460L	M	L	NA	[129]
M460T	M	T	R (9.3)	[96, 101]
M460V	**M**	**V**	**R (8.3)**	**[74, 75, 82, 93, 96, 99, 100, 108, 112, 116, 122, 134, 137, 138, 142, 145, 153, 155, 170-172]**
V466G	V	G	R (3.5)	[96, 122]
C480R	D	R	R (9)	[118]
C518Y	C	Y	R (12)	[123]
H520Q	**H**	**Q**	**R (10)**	**[74, 96, 108, 134, 137, 138, 153]**
P521L	P	L	R (17)	[117]
del591-594 [b]	ACRA	del 4	R (6**)**	[96, 108, 137, 173]
del590-600	AACRALENGKL	del 11	R (6.3)	[119]
del590-603	AACRALENGKL THC	del 14	NA	[174]
del591-607	ACRALENGKLT HCSDAC	del 17	R (6.2)	[96, 108]
C592F	C	F	R (31.5)	[120]
C592G	**C**	**G**	**R (2.9)**	**[73, 74, 93, 94, 96, 101, 108, 111, 112, 116, 122, 138, 141, 145, 153, 155, 157]**
A594E	A	E	R (3.0)	[96, 101]
A594G	A	G	R (13.5)	[124]
A594P	A	P	R (2.9)	[73, 96, 113, 134, 145, 158]
A594T	A	T	R (2.7)	[73, 94, 96, 99, 108, 116, 134, 137, 138, 167, 170]
A594V	**A**	**V**	**R (8.3)**	**[73, 74, 82, 83, 94, 96, 99, 101, 103, 104, 108, 112, 116, 134, 137, 138, 140, 145, 153, 155, 158, 159, 167, 170-172]**
del594-595	AL	del 2	NA	[131]
L595F	**L**	**F**	**R (15.7)**	**[96, 108, 116, 122, 145, 163, 171]**
L595S	**L**	**S**	**R (9.2)**	**[73-75, 83, 94, 96, 99, 104, 108, 112, 116, 134, 137, 138, 145, 153, 157, 159, 167, 170-172]**
L595T	L	T	R (2.2)	[116]

L595W	**L**	**W**	**R (5.1)**	**[83, 96, 108, 123, 134, 153]**
del595	L	del 1	R (13.3)	[96, 153, 175]
del595-603	LENGKLTHC	del 9	R (8.4)	[96, 114, 176]
del596	E	del 1	NA	[132]
E596G	E	G	R (2.3)	[73, 95, 96, 108]
E596Y	E	Y	R (6.4)	[95]
G598S	G	S	NA	[96, 127, 177]
K599E	K	E	NA	[132]
K599T	K	T	R (5.3)	[96, 115]
del600	L	del 1	r (1.9)	[96, 108, 153]
del601	T	del 1	NA	[128, 153]
del601-603	THC	del 3	R (11)	[96, 142]
T601M	T	M	NA	[133]
C603R	C	R	R (3.6 - 8.3)	[96, 101, 122, 134]
C603S	C	S	r (1.9)	[96, 101, 134]
C603W	**C**	**W**	**R (8)**	**[73, 74, 96, 99, 101, 104, 108, 111, 116, 134, 137, 145, 153, 157]**
C607F	C	F	r (1.9)	[74, 96, 108, 137]
C607T	C	T	R (12.5)	[121]
C607Y	C	Y	R (12.5)	[96, 108, 134, 137, 140, 144]
I610T	I	T	R (2.6)	[95]
A613V	A	V	R (2.3)	[125]
del617	Y	del 1	R (10)	[118]
E655K	E	K	r (1.7)	[126]

Boldface indicates the most common mutations conferring resistance; Deletions start at the designated codon and continue through the number of shown codons; R, resistant strain (≥2 fold reduction in susceptibility to GCV/VGCV); r, low-grade resistance or <2 fold reduction in susceptibility GCV/VGCV; NA, not available.

a) The level of resistance of each mutation is expressed as the ratio of the half maximal inhibitory concentration (IC_{50}) of the mutant to that of drug – sensitive wild-type (IC_{50} of mutant/IC_{50} of wild type ratio); b) Deletion of codons 591 to 594 results in the same mutant virus as deletion of codons 590 to 593.

Attachment II: Mutations in DNA polymerase (*UL54*) gene associated with antiviral resistance in laboratory and clinical isolates confirmed by marked transfer.

Mutation	Amino acid change		Viral Phenotype [a]			References
	Wild Type (Wt)	Mutant	GCV/ VGCV (IC_{50})	CDV (IC_{50})	FOS (IC_{50})	
Exo I (295 - 312)						
D301N	D	N	**R (2.6)**	**R (3)**	S (0.5)	[96, 143]
Regions IV / Exo II (379 - 421 / 404 - 418)						
N408D	N	D	**R (4.9)**	**R (5.6)**	S (1.3)	[96, 105, 116, 138, 140, 151]
N408K	N	K	**R (4.2)**	**R (21)**	S (0.7)	[82, 96, 145, 160]
N408S	N	S	**R (3.1)**	**R (7.5)**	S (1.0)	[144]
N410K	N	K	**R (2.9)**	**R (3)**	S (0.8)	[96, 143]
F412C	F	C	**R (4.2)**	**R (18)**	S (1.2)	[74, 96, 111, 116, 151, 157]
F412L	F	L	**R (4.6)**	**R (9.4)**	S (1.1)	[96, 98, 161]
F412S	F	S	**R (5.3)**	**R (13)**	S (0.8)	[96, 98, 134, 161]
F412V	F	V	**R (4.3)**	**R (15.5)**	S (1.1)	[96, 151]
D413A	D	A	**R (6.5)**	**R (11)**	S (0.8)	[96, 142]
D413E	D	E	**R (4.8)**	**R (4.3)**	S (0.8)	[96, 138, 143, 145]
D413N	D	N	**R (3.8)**	**R (10)**	S (1.0)	[140]
Outside the conserved regions						
P488R	P	R	**R (3.5)**	**R (7.9)**	S (0.6)	[98, 105]
∂-Region C (492 - 588)						
N495K	N	K	S (1.1)	S (1.1)	**R (3.4)**	[75, 96, 154]
K500N	K	N	**R (3.2)**	**R (3.0)**	S (1.2)	[98, 105]
L501I	L	I	**R (6)**	**R (9.1)**	S (1.4)	[74, 82, 96, 116, 151]
T503I	T	I	**R (2.9)**	**R (6.1)**	S (0.5)	[74, 96, 116, 143]
A505V	A	V	**r (1.8)**	**R (2)**	S (1.1)	[146]
K513E	K	E	**R (5)**	**R (9.1)**	S (1.4)	[96, 116, 151, 157]
K513N	K	N	**R (6)**	**R (12.5)**	S (1.5)	[96, 164]
K513R	K	R	**R (3.7)**	**R (10)**	S (1.1)	[116, 140]
D515E	D	E	**R (2.7)**	NT	**R (4.6)**	[145]
D515Y	D	Y	**R (5.6)**	NT	**R (4.6)**	[145]
L516R	L	R	**R (2.1)**	**R (5.1)**	S (0.8)	[96, 143]
I521T	I	T	**R (2.1)**	**R (5.1)**	S (0.8)	[96, 104, 162]
P522A	P	A	**R (3)**	**R (4.1)**	S (1)	[74, 96, 138, 162]
P522S	P	S	**R (3.1)**	**R (3.6)**	S (1.1)	[74, 75, 96, 99, 134, 140, 151, 153, 156]
del524	C	del 1	**R (3.5)**	**R (9.7)**	S (1.1)	[144]
V526L	V	L	**R (5.5)**	**R (2.5)**	S (1.8)	[126]
ExoIII / ∂ region C (533 - 545 / 492 - 588)						
C539G	C	G	**R (3.1)**	**R (4.4)**	S (1.0)	[140]
C539R	C	R	**R (3.2)**	**R (13.3)**	S (0.7)	[98, 105]
D542E	D	E	S (1.5)	**R (12)**	S (1.7)	[148]
L545S	L	S	**R (3.5)**	**R (9.1)**	S (1.2)	[73, 96, 105, 139, 151]
L545W	L	W	**R (4.9)**	**R (6.3)**	S (1.3)	[96, 98, 161]

∂-Region C (492 – 588)						
T552N	T	N	**r (1.9)**	S (1.2)	**R (2.6)**	[98, 105]
Q578H	Q	H	**R (3.3)**	**R (2.3)**	**R (4.5)**	[96, 98, 103, 140, 161, 178]
Q578L	Q	L	**r (1.9)**	S (0.8)	**R (3.0)**	[97, 140]
S585A	S	A	S (1.5)	S (1.4)	**R (2.7)**	[98, 105]
D588E	D	E	S (1.3)	S (1.1)	**R (2.3)**	[96, 116, 151]
D588N	D	N	**R (3.8)**	**R (2.7)**	**R (3.2-9)**	[74, 96, 155, 157, 161]
Outside the conserved regions						
F595I	F	I	S (1.3)	S (1.2)	**R (2.0)**	[98, 105]
A692S	A	S	S (1.6)	S (1.7)	**R (3.3)**	[143, 153]
Region II (696-742)						
T700A	T	A	S (0.9)	S (1.5)	**R (4.7)**	[93, 96, 138, 151, 159]
V715M	V	M	S (1.0)	S (1.1)	**R (5.5)**	[94, 96, 134, 140, 151, 156, 158, 159]
I726T	I	T	**R (2.0)**	**r (1.7)**	S (1.1)	[146]
I726V	I	V	**r (1.9)**	**r (1.9)**	S (1.2)	[146]
Outside the conserved regions						
E756D	E	D	S (1.2)	S (0.7)	**R (3.4)**	[96, 138, 143]
E756K	E	K	**R (2.5)**	**R (2.2)**	**R (>8)**	[96, 143, 157]
E756Q	E	Q	S (1.7)	S (1.0)	**R (4.3)**	[96, 156]
Region VI (771- 790)						
L773V	L	V	**R (3.0)**	**R (2.5)**	**R (4.4)**	[140]
L776M	L	M	**R (2.5)**	S (1)	**R (3.5)**	[96]
W780V	W	V	S (1.5)	NA	**r (1.9)**	[149]
V781I	V	I	**R (1-4)**	S (1.2)	**R (4-5.2)**	[96, 116, 140, 151, 153, 156, 157]
V787A	V	A	**R (2.5)**	NA	**R (3.5)**	[158]
V787L	V	L	**R (2.4)**	S (1)	**R (4.1)**	[96, 105, 153, 156]
Outside the conserved regions						
L802M	L	M	**R (1.1-3.5)**	S (0.9-1.8)	**R (3.2-11)**	[74, 96, 104, 105, 111, 116, 145, 151, 155, 157]
L802V	L	V	**r (1.8)**	S (1.1)	S (0.9)	[98, 105]
Region III (805 – 845)						
K805Q	K	Q	S (1)	**R (2.2)**	S (0.18)	[96, 116, 151, 152]
A809V	A	V	**R (2.6)**	S (1.7)	**R (6.3)**	[96, 134, 140, 141]
V812L	V	L	**R (2.5)**	**R (3.2)**	**R (2.9)**	[96, 105, 155, 164]
T813S	T	S	**R (2.5)**	**R (2.7)**	**R (4.9)**	**[**96, 141]
T821I	T	I	**R (4.5)**	**r (1.9)**	**R (21)**	[96, 116, 151, 152]
P829S	P	I	**R (2.0)**	S (1.6)	S (1.1)	[98, 105]
A834P	A	P	**R (5.4)**	**R (3)**	**R (6.4)**	[74, 82, 96, 103, 140, 145, 160]
T838A	T	A	S (1.8)	S (0.8)	**R (2.4)**	[96, 155]
G841A	G	A	**R (3.2)**	**R (2.6)**	**R (4.3)**	[74, 96, 138, 141]
G841S	G	S	**R (2.2)**	S (1.1)	**R (2.1)**	[146]
M844T	M	T	S (1.4)	S (1.3)	**R (2.5)**	[150]
M844V	M	V	**R (2.5)**	**r (1.6)**	**R (2.2)**	[150]
Outside the conserved regions						
L862F	L	F	**r (1.7)**	S (0.9)	S (1.1)	[98, 105]
V946L	V	L	S (1.1)	S (0.9)	**R (2.4)**	[98, 105]
L957F	L	F	**R (2.7)**	S (1.4)	S (1.3)	[98, 105]

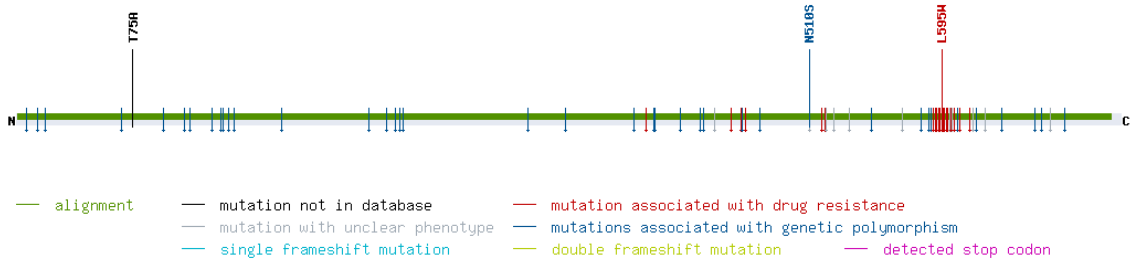
Region V (978 – 988)						
del981-2	DL	del 2	**R (8.3)**	**R (2.8)**	**R (3.6)**	[96, 112, 140, 143, 163]
A987G	A	G	**R (5.3)**	**R (11.3)**	S (1.2)	[74, 82, 96, 134, 145, 151, 153]

Boldface indicates the mutations conferring resistance. Deletions start at the designated codon and continue through the number of shown codons; R, resistant strain (≥2 fold reduction in susceptibility to antiviral drugs); r, low-grade resistance or <2 fold reduction in susceptibility to antiviral drugs; S, sensitive strain; NA, not available; NT, not tested.

a) The level of resistance of each mutation is expressed as the ratio of the half maximal inhibitory concentration (IC_{50}) of the mutant to that of drug – sensitive wild-type (IC_{50} of mutant/IC_{50} of wild type ratio);

Attachment III: *UL97* sequencing analysis of codon 1 until 707 in comparison to the sequence information of the Merlin strain, for the 22 patients included in this study.

Patient 1



List of Mutations:

- **Resistance Mutation:** L595W
- **Polymorphism:** N510S
- **Unknown mutation:** T75A

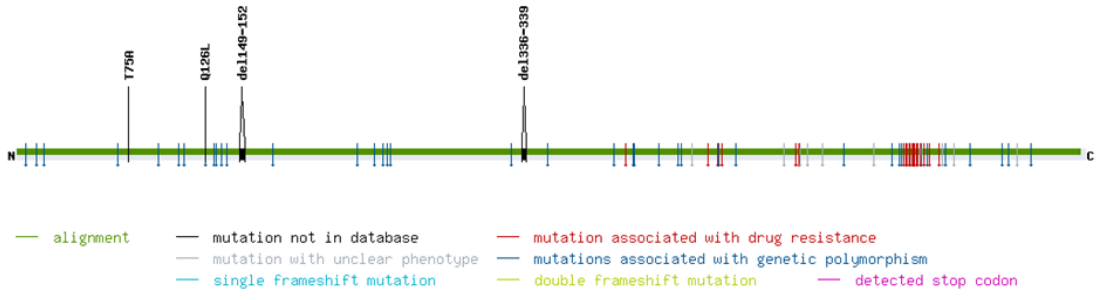
Patient 2

List of Mutations:

- **Unknown mutation:** T75A

Missing information of codon: del 149-151

Patient 3

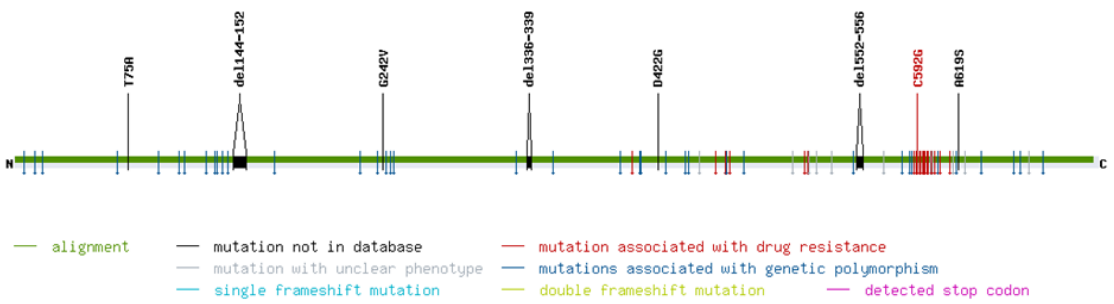


List of Mutations:

- **Polymorphism**: Q126L
- **Unknown mutation:** T75A

Missing information of codon: del 149-152, del 336-339

Patient 4

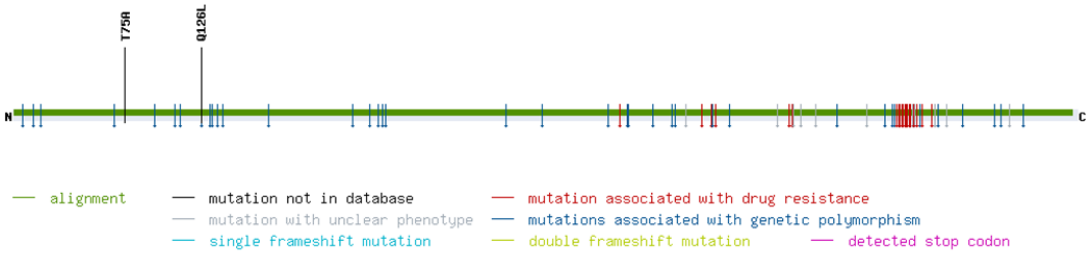


List of Mutations:

- **Resistance Mutation:** C592G
- **Unknown mutations:** T75A, G242V, D422G, A619S

Missing information of codon: del 144-152, del 336-341, del 552-556

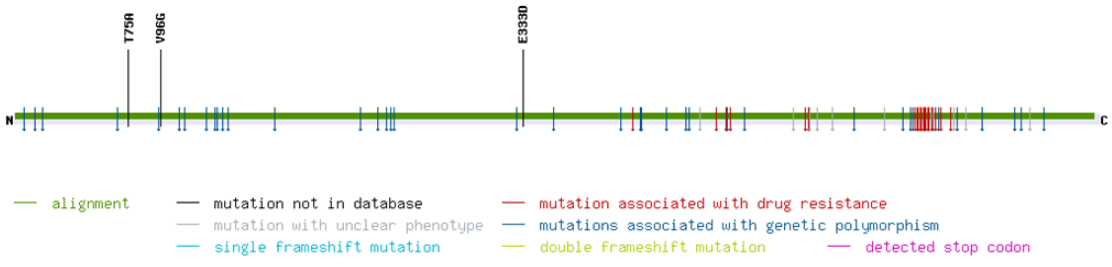
Patient 5



List of Mutations:

- **Polymorphism:** Q126L
- **Unknown mutation:** T75A

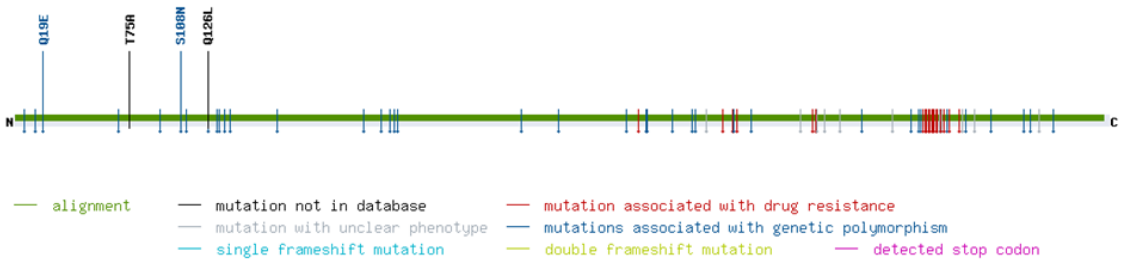
Patient 6



List of Mutations:

- **Unknown mutations:** T75A, V96G, E333D

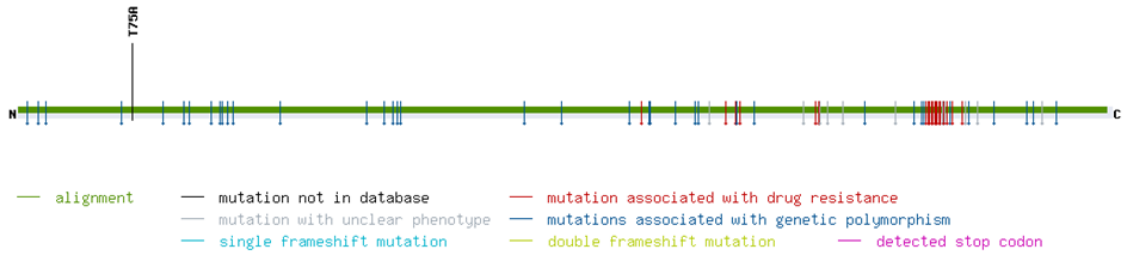
Patient 7



List of Mutations:

- **Polymorphisms:** Q19E, S108N, Q126L
- **Unknown mutation:** T75A

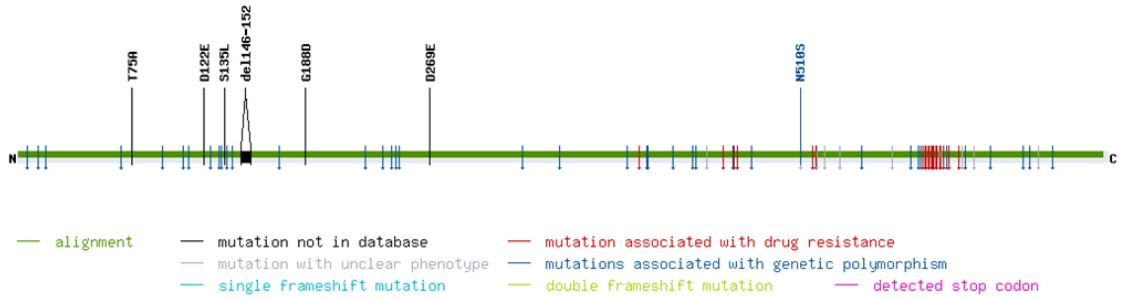
Patient 8



List of Mutations:

- **Unknown mutations:** T75A

Patient 9

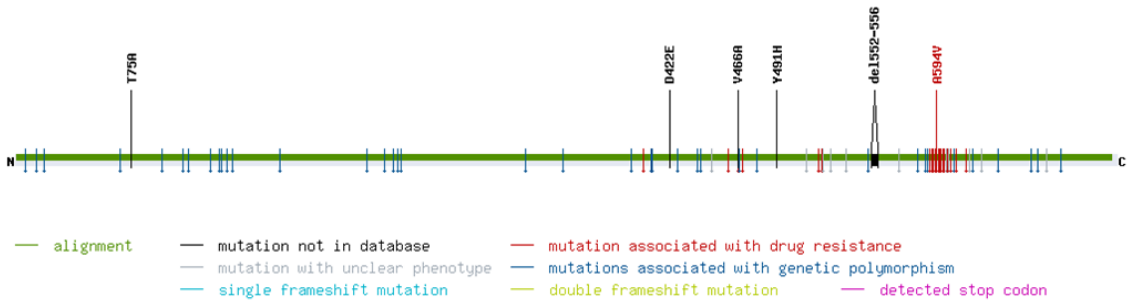


List of Mutations:

- **Polymorphism:** N510S
- **Unknown mutations:** T75A, D122E, S135L, G188D, D269E

Missing information of codon: del 146-152

Patient 10

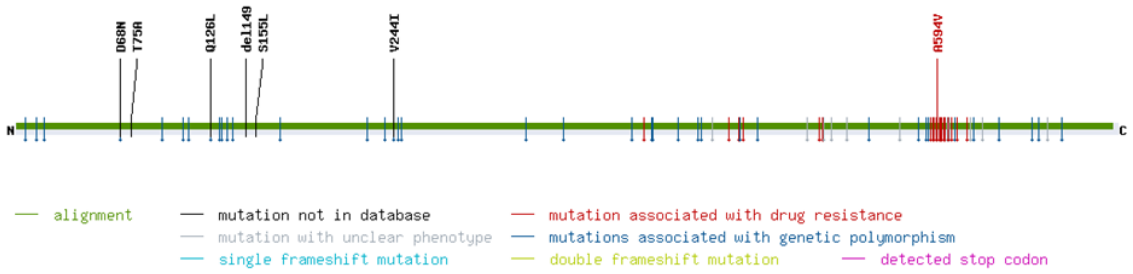


List of Mutations:

- **Resistance Mutation:** A594V
- **Unknown mutations:** T75A, D422E, V466A, Y491H

Missing information of codon: del 552-556

Patient 11

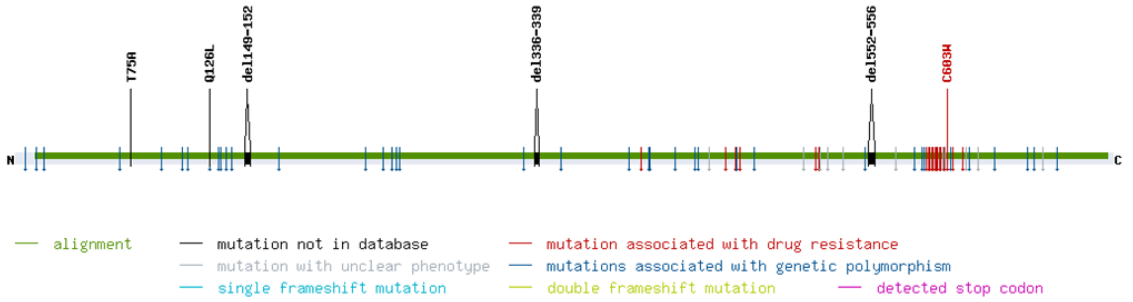


List of Mutations:

- **Resistance Mutation:** A594V
- **Polymorphisms:** D68N, Q126L, V244I
- **Unknown mutations:** T75A, S155L

Missing information of codon: del 149

Patient 12

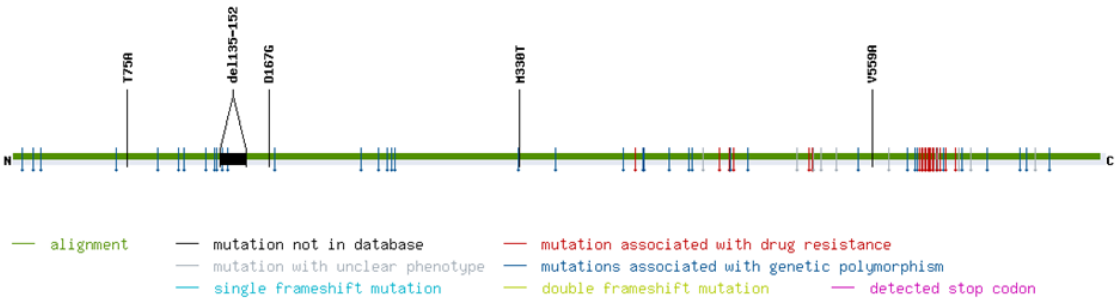


List of Mutations:

- **Resistance Mutation:** C603W
- **Polymorphism:** Q126L
- **Unknown mutation:**T75A

Missing information of codon: del 149-152, del 336-339, del 552-556

Patient 13



List of Mutations:

- **Unknown mutation:** T75A, D167G, M330T , V559A

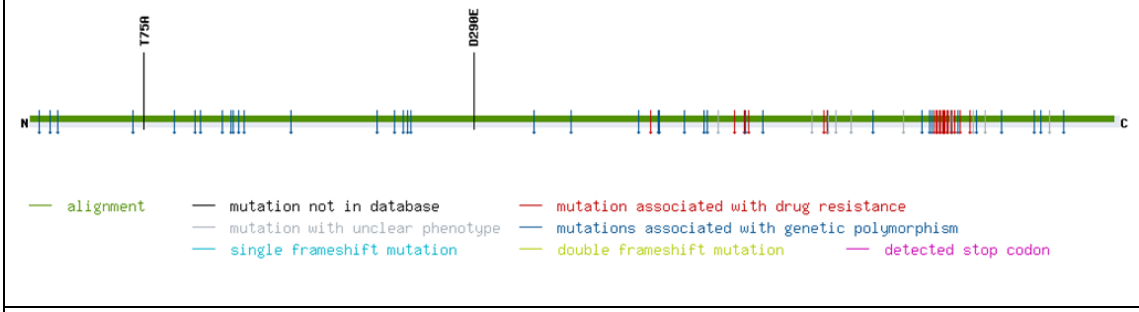
Missing information of codon: del 135-152

Patient 14

List of Mutations:

- **Polymorphisms:** Q19E, S108N, Q126L
- **Unknown mutation:** T75A

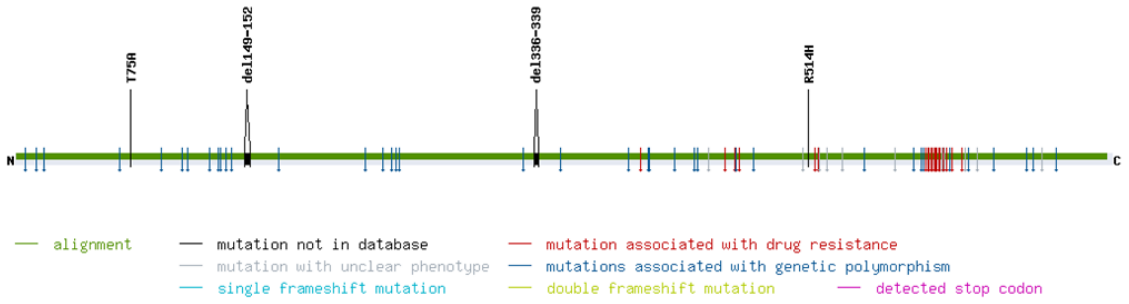
Patient 15



List of Mutations:

- **Unknown mutations:** T75A, D290E

Patient 16

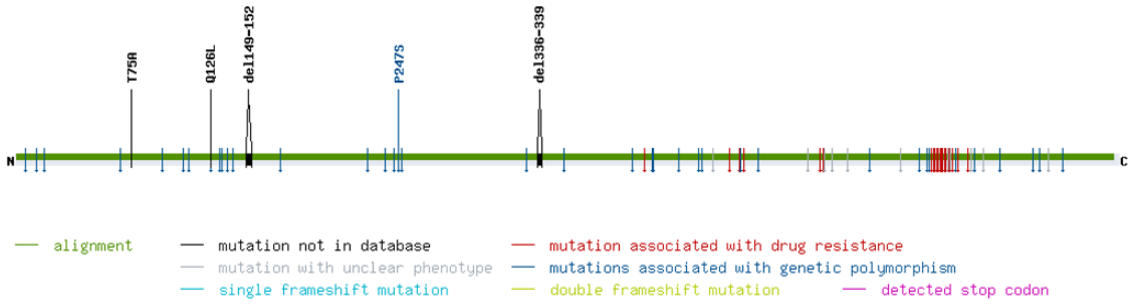


List of Mutations:

- **Unknown mutations:** T75A, R514H

Missing information of codon: del 149-152, del 336-339

Patient 17

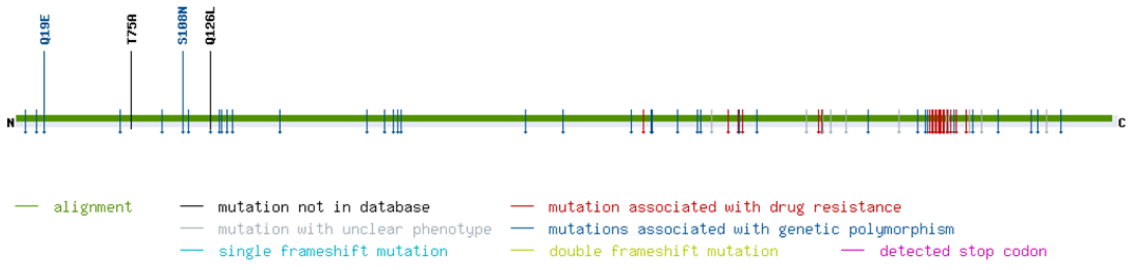


List of Mutations:

- **Polymorphisms:** Q126L, P247S
- **Unknown mutation:** T75A

Missing information of codon: del 149-152, del 336-339

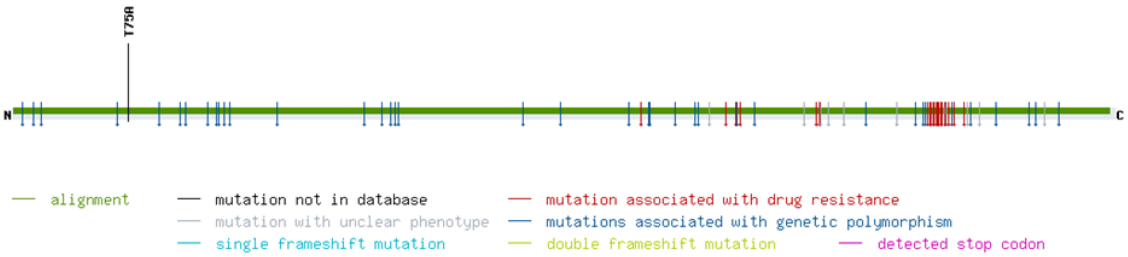
Patient 18



List of Mutations:

- **Polymorphisms:** Q19E, S108N, Q126L
- **Unknown mutation:** T75A

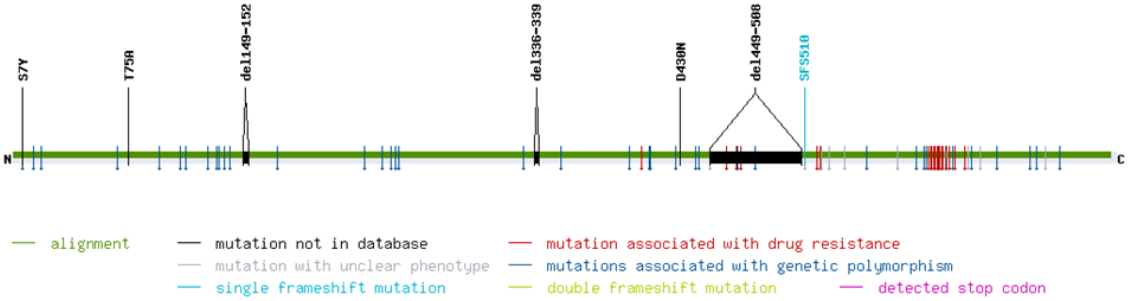
Patient 19



List of Mutations:

- **Unknown mutation:** T75A

Patient 20

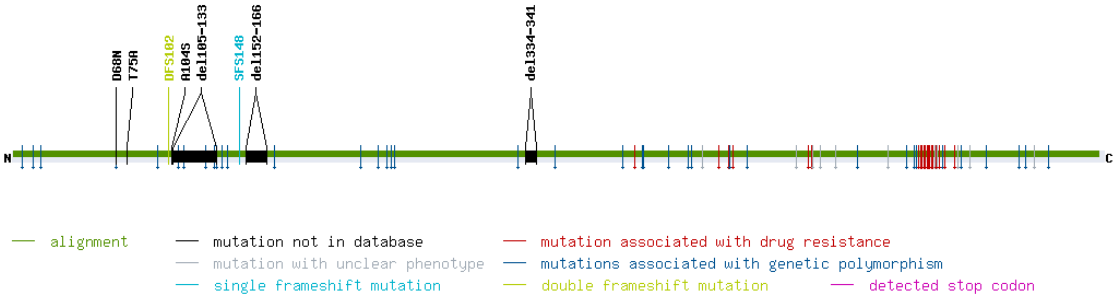


List of Mutations:

- **Unknown mutations:** S7Y, T75A, D430N

Missing information of codon: del 149-152, del 336-339, del 449-50

Patient 21

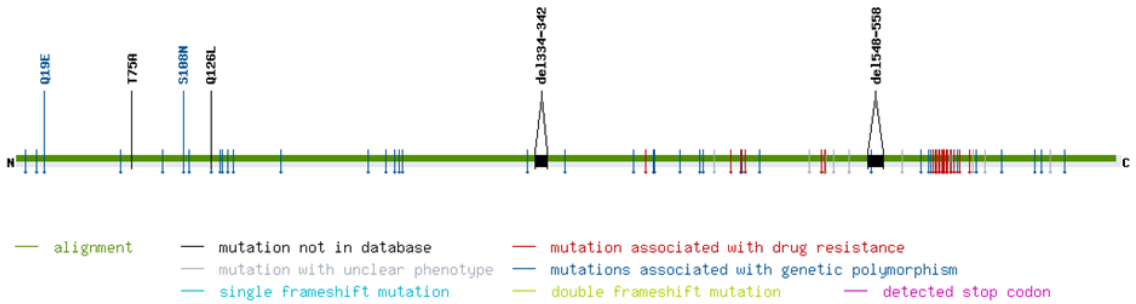


List of Mutations:

- **Polymorphism:** D68N
- **Unknown mutations:** T75A, A104S

Single frameshift mutation: SFS148
Double frameshift mutation: DFS102
Missing information of codon: del 105-133, del 152-166, del 334-341

Patient 22



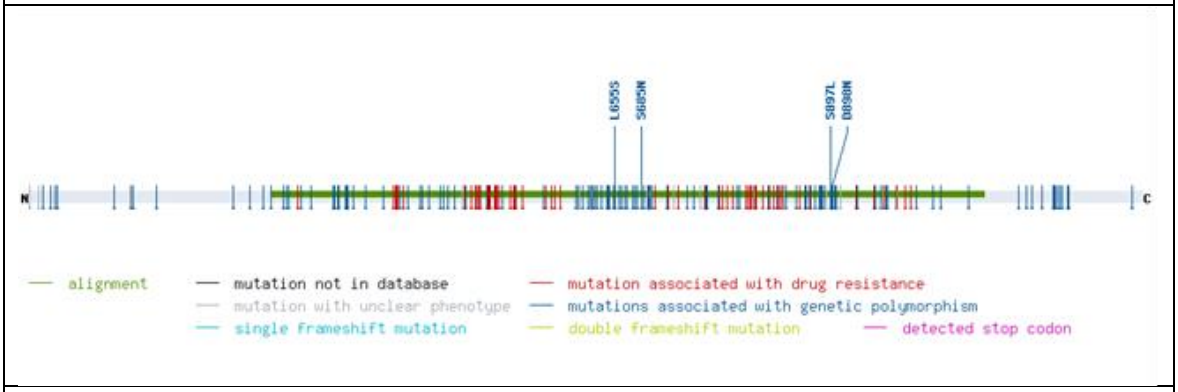
List of Mutations:

- **Polymorphisms:** Q19E, S108N, Q126L
- **Unknown mutations:** T75A

Missing information of codon: del 334-342, del 548-558

Attachment IV: *UL54* sequencing analysis of codon 272 until 1069 in comparison to the sequence information of the TB40/E strain, for the 22 patients included in this study.

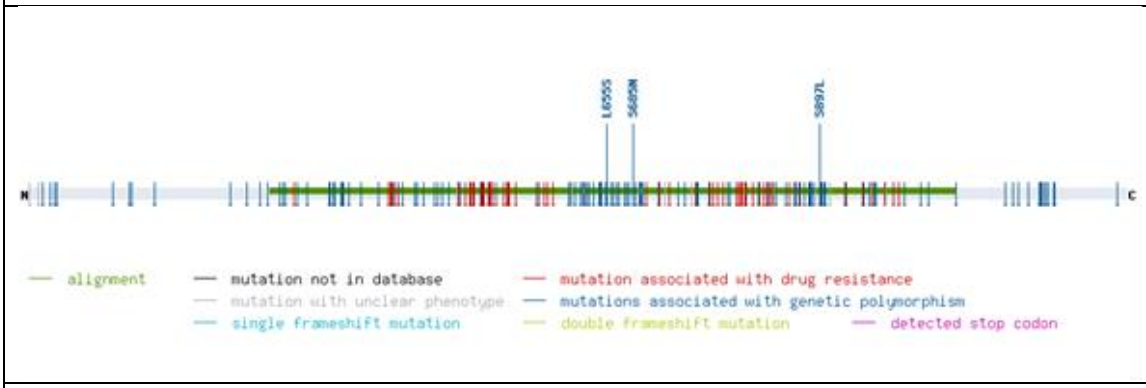
Patient 1



List of Mutations:

- **Polymorphisms:** L655S, S685N, S897L, D898N

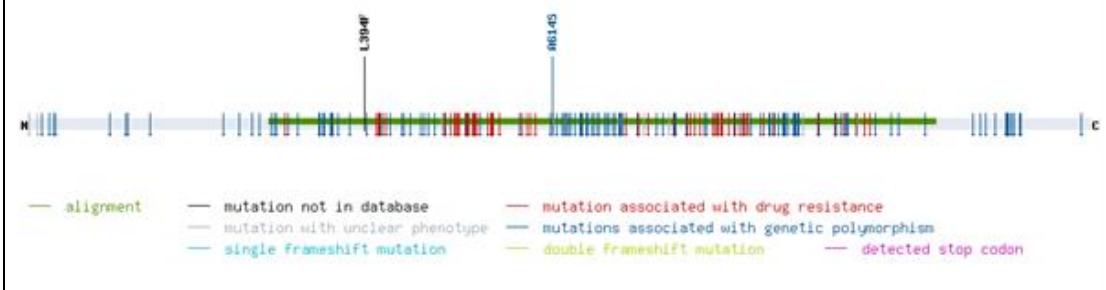
Patient 2



List of Mutations:

- **Polymorphisms:** L655S, S685N, S897L

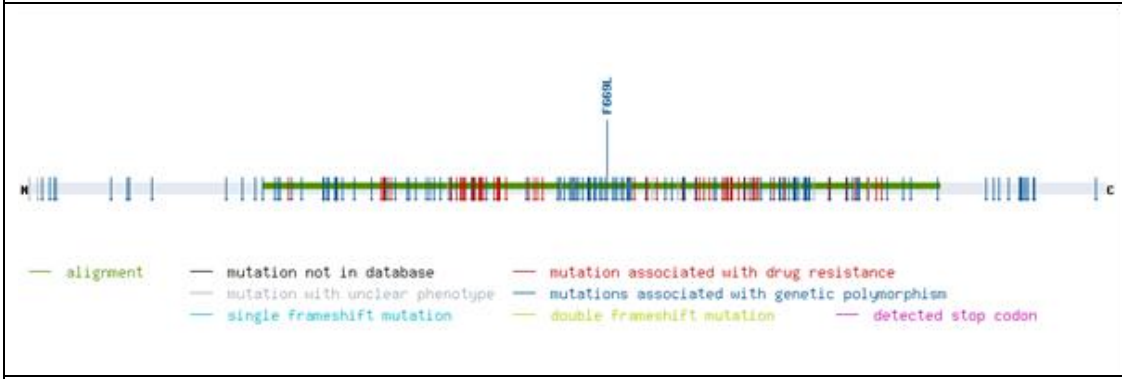
Patient 3



List of Mutations:

- **Polymorphism:** A614S
- **Unknown mutation:** L394F

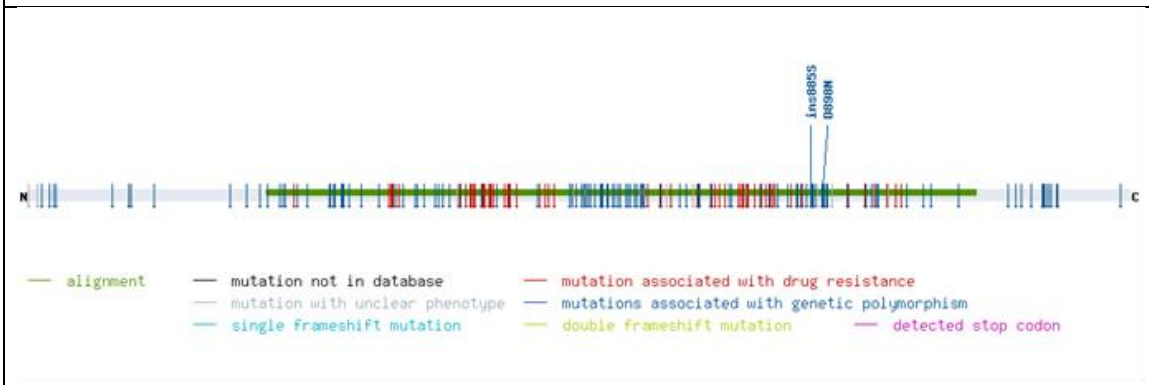
Patient 4



List of Mutations:

- **Polymorphism:** F669L

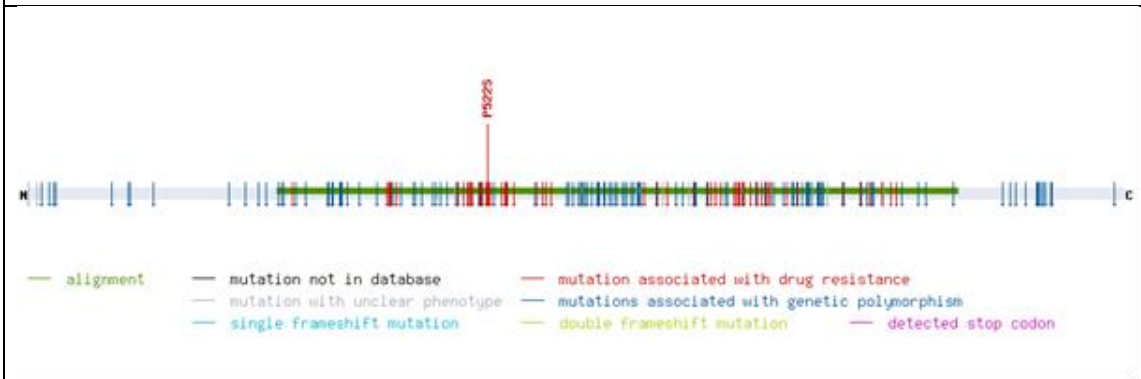
Patient 5



List of Mutations:

- **Polymorphisms:** ins885S and D898N

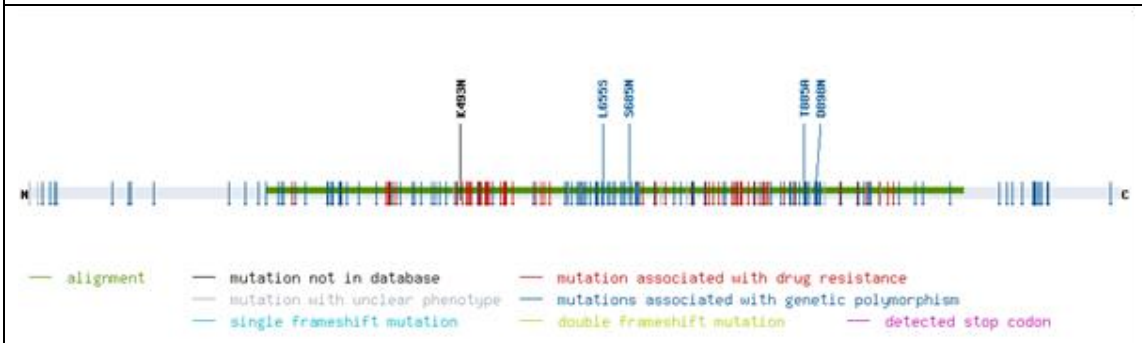
Patient 6



List of Mutations:

- **Resistance mutation:** P522S

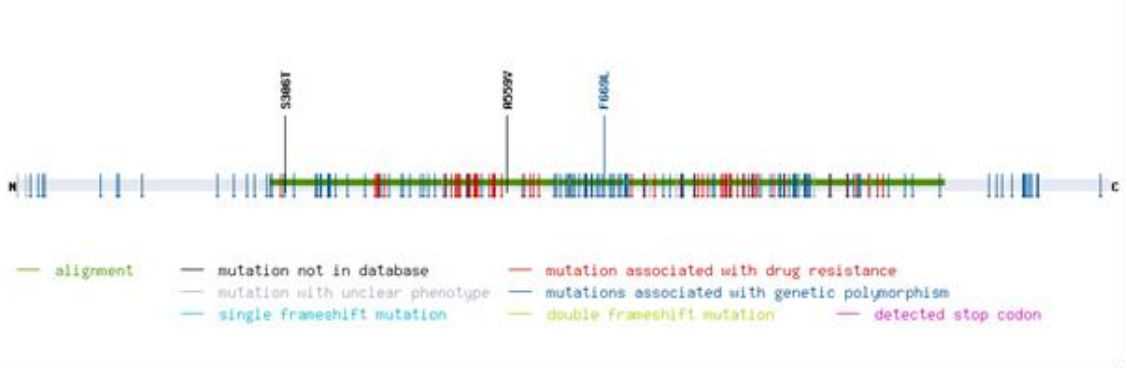
Patient 7



List of Mutations:

- **Polymorphisms:** L655S, S685N, T885A, D898N
- **Unknown mutation:** K493N

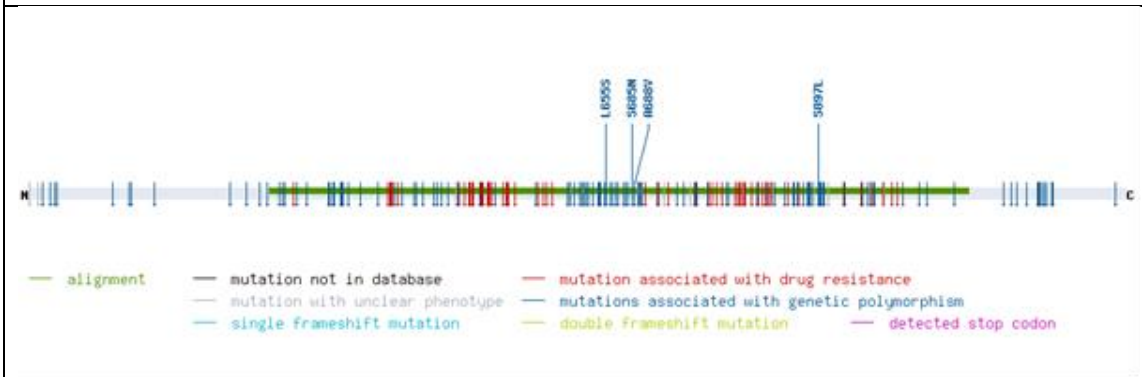
Patient 8



List of Mutations:

- **Polymorphism:** F669L
- **Unknown mutations:** S306T, A559V

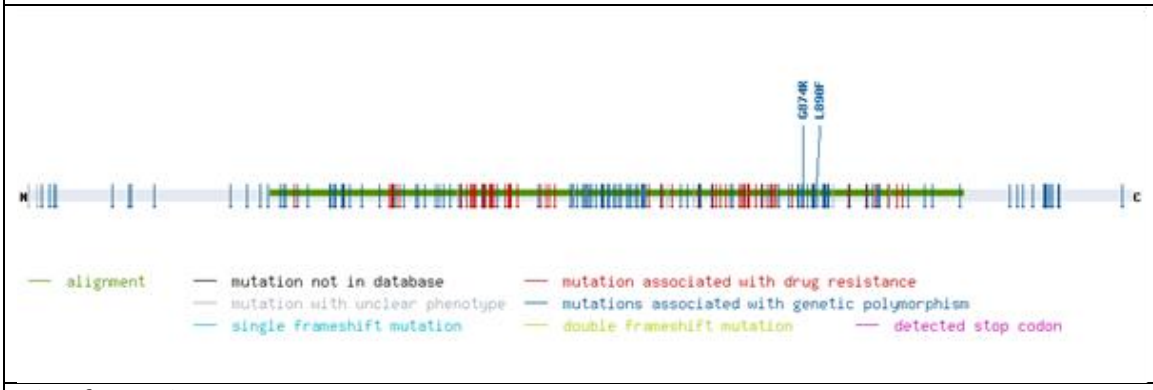
Patient 9



List of Mutations:

- **Polymorphisms:** L655S, S685N, A688V, S897L

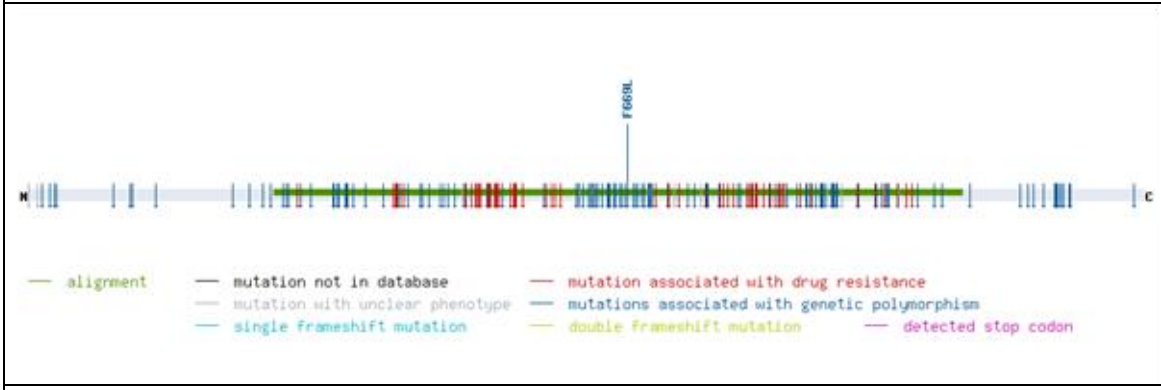
Patient 10



List of Mutations:

- **Polymorphisms:** G874R, L890F

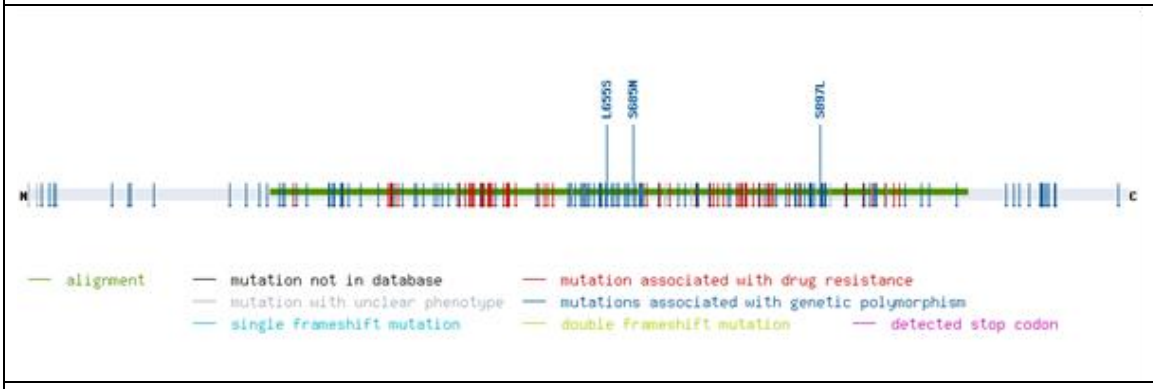
Patient 11



List of Mutations:

- **Polymorphism:** F669L

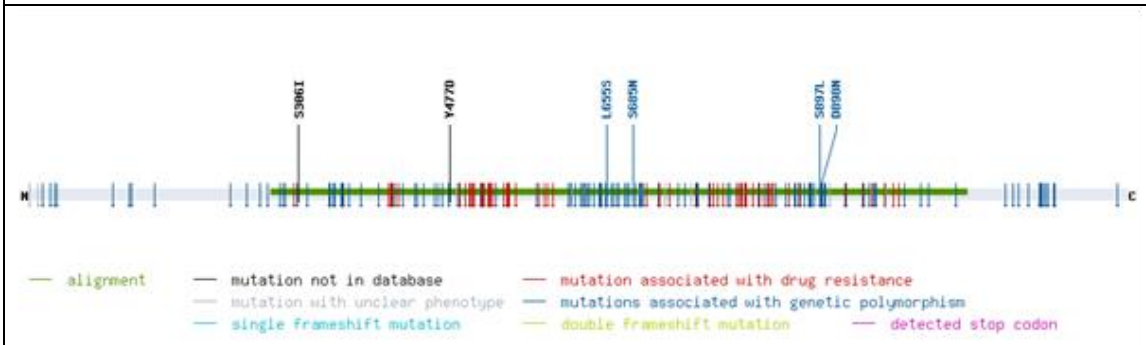
Patient 12



List of Mutations:

- **Polymorphisms:** L655S, S685N, S897L

Patient 13



List of Mutations:

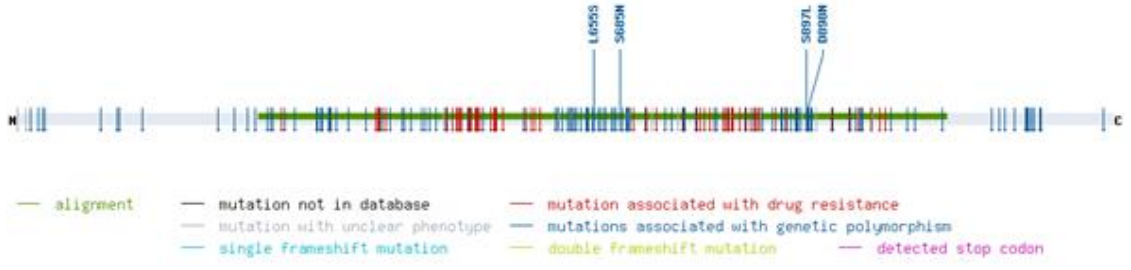
- **Polymorphisms:** L655S, S685N, S897L, D898N
- **Unknown mutations:** S306I, Y477D

Patient 14

List of Mutations:

- **Polymorphisms:** L655S, S676G, S685N, S897L
- **Unknown mutation:** L845V

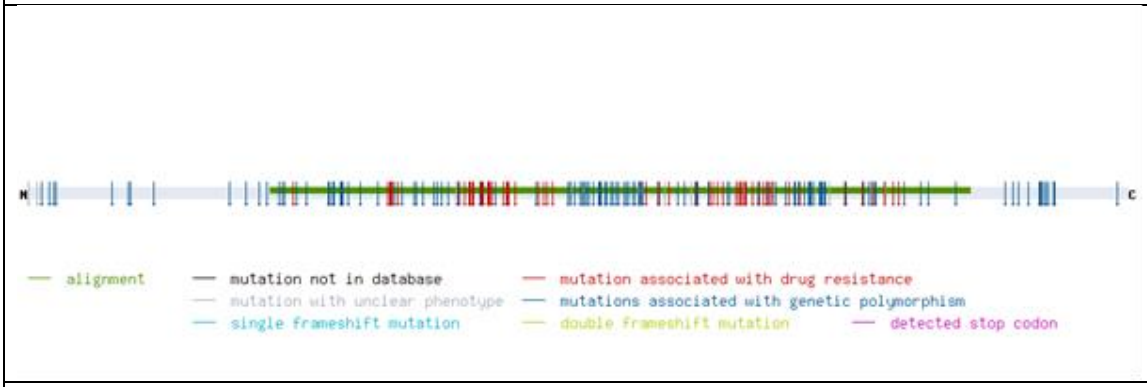
Patient 15



List of Mutations:

- **Polymorphisms:** L655S, S685N, S897L, D898N

Patient 16



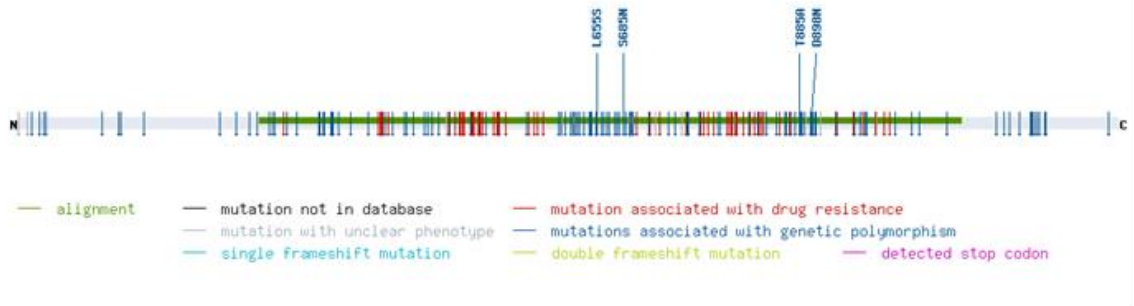
List of Mutations: No Mutations found

Patient 17

List of Mutations:

- **Polymorphisms:** L655S, S685N, S897L

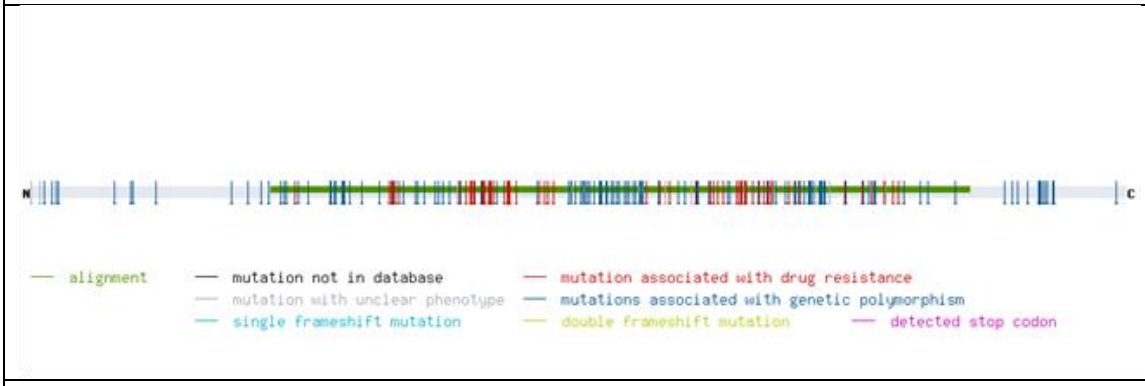
Patient 18



List of Mutations:

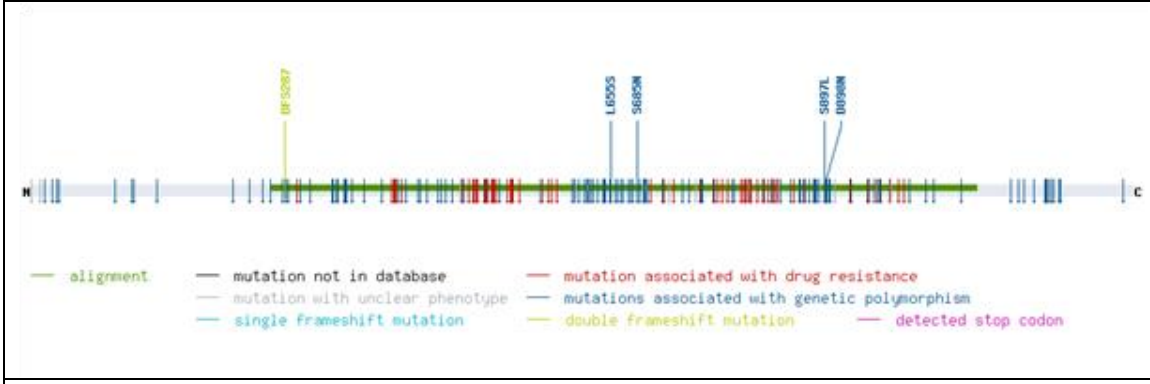
- **Polymorphisms:** L655S, S685N, T885A, D898N

Patient 19



List of Mutations: No mutations found.

Patient 20



List of Mutations:

- **Polymorphisms:** L655S, S685N, S897L , D898N

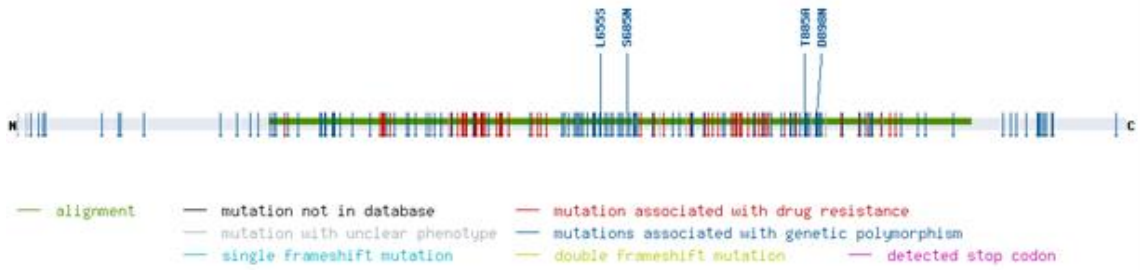
Double frameshift mutation: DFS287

Patient 21

List of Mutations:

- **Resistance mutation:** L957F
- **Polymorphisms:** G874R, L890F

Patient 22



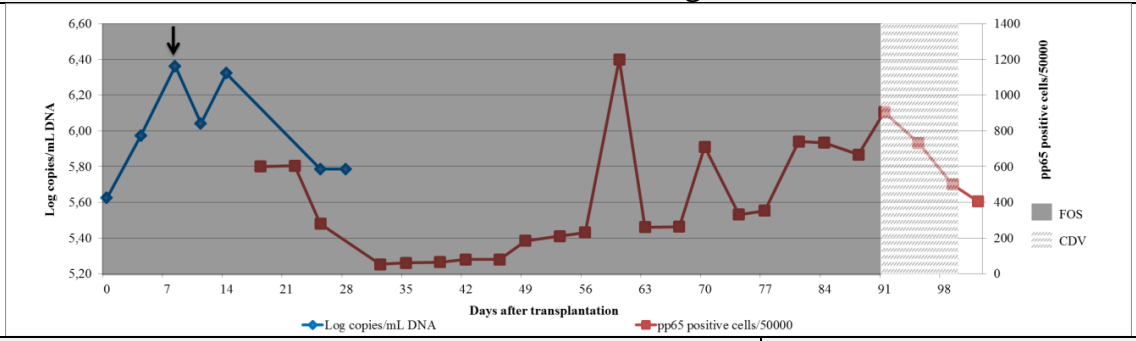
List of Mutations:

- **Polymorphisms:** L655S, S685N, T885A, D898N

Attachment V: HCMV monitoring, demographic and clinicopathological characteristics, and antiviral treatment of 22 patients. The arrows point to the sample that was selected for mutations analysis.

Patient 1

HCMV Monitoring

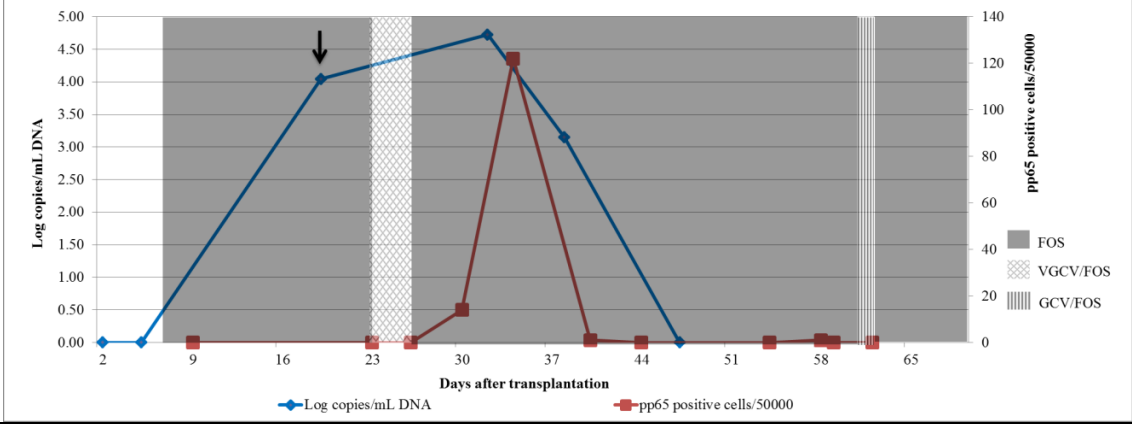


Characteristics of patient		Antiviral Treatment
Gender	Male	**Day +0** – FOS 288 mg 8h/8h 35 days; **Day +41** – FOS 648 mg 1x/day 19 days; **Day +60** – FOS 432 mg 8h/8h 28 days; **Day +89** – FOS 648 mg 1x/day 2 day; **Day +91** – CID 35 mg 1x/day 8 days.
Age (months old)	< 12 months	
Underlying disease	Primary immunodeficiency	
Conditioning regimen	Myeloblative	
Stem cell source	Cord Blood	
HLA status	Mismatch or Unrelated	
HCMV D/R status	D-/R+	

HLA, Human Leukocyte Antigen; HCMV, Human cytomegalovirus; D, Donor; R, Recipient; D-, Negative Donor; R+, Positive recipient

Patient 2

HCMV Monitoring

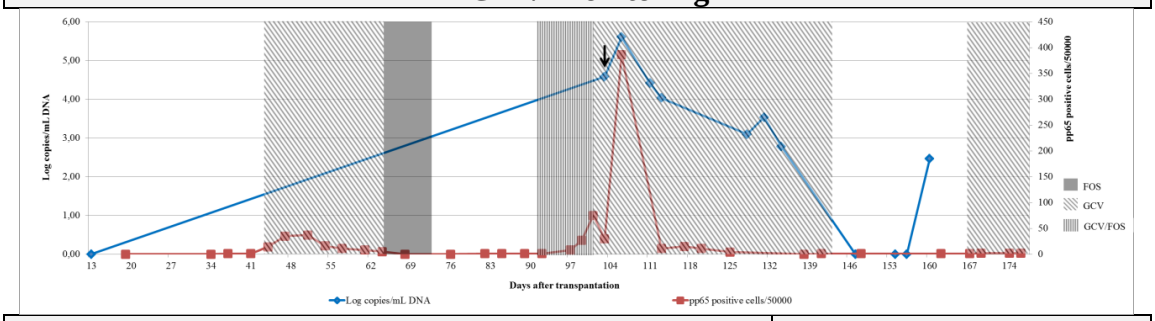


Characteristics of patient		Antiviral treatment
Gender	Male	**Day +7** – FOS 3360 mg 8h/8h 44 days; **Day +23** – VGCV 900 mg 12/12h 3 days; **Day +52** – FOS 4850 mg 1x/day 17 days; **Day +58** – FOS 4850 mg 1x/day 2 days; **Day +60** – GCV 70 mg 1x/day 2 days.
Age (years old)	24	
Underlying disease	paroxysmal nocturnal hemoglobinuria	
Conditioning regimen	Myeloblative	
Stem cell source	Peripheral Blood	
HLA status	Match/Related	
HCMV D/R status	D+/R+	

HLA, Human Leukocyte Antigen; HCMV, Human cytomegalovirus; D, Donor, R; Recipient; D+, Positive Donor; R+, Positive recipient

Patient 3

HCMV Monitoring

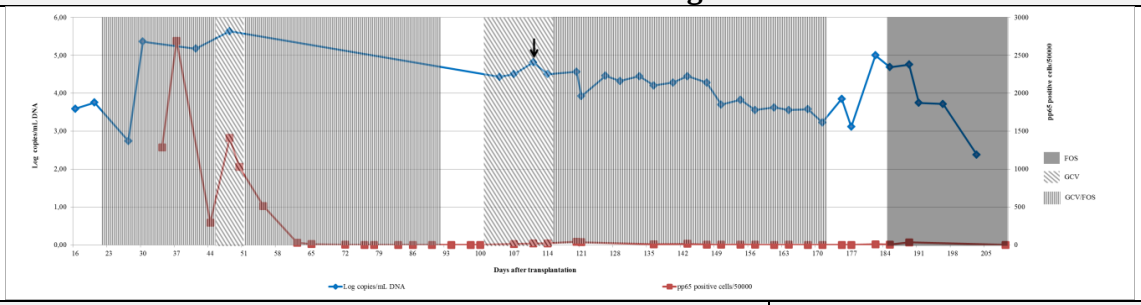


Characteristics of patient		Antiviral treatment
Gender	Female	**Day +44** – GCV 100 mg 2x/day 21 days; **Day +65** – FOS 1300 mg 8h/8h 7 days; **Day +97** – FOS 1920 mg 1x/day 4 days; **Day +97** – GCV 100 mg 12h/12h 45 days; **Day +167** – GCV 100 mg 12h/12h 1 day; **Day +169** – GCV 50 mg 12h/12h 8 days.
Age (years old)	7	
Underlying disease	Acute Myeloid Leukemia	
Conditioning regimen	Reduced intensity	
Stem cell source	Peripheral Blood	
HLA status	Mismatch or Unrelated	
HCMV D/R status	D-/R+	

HLA, Human Leukocyte Antigen; HCMV, Human cytomegalovirus; D, Donor; R, Recipient, D-, Negative Donor; R+, positive recipient

Patient 4

HCMV Monitoring

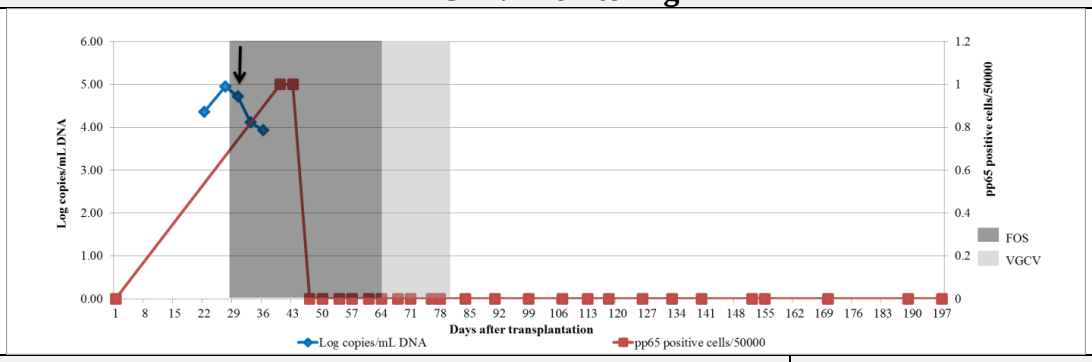


Characteristics of patient		Antiviral treatment
Gender	Male	**Day +22** – GCV 44 mg 12h/12h 23 days; **Day +22** – FOS 504 mg 8h/8h 23 days; **Day +35** – GCV 43 mg 12h/12h 57 days; **Day +51** – FOS 520 mg 8h/8h 41 days; **Day +101** – GCV 40 mg 12h/12h 70 days; **Day +114** – FOS 480 mg 8h/8h 57 days; **Day +185** – FOS 508 mg 8h/8h 32 days.
Age (years old)	1	
Underlying disease	Acute lymphoid leukemia B lineage	
Conditioning regimen	Myeloblative	
Stem cell source	Cord Blood	
HLA status	Mismatch or Unrelated	
HCMV D/R status	D-/R+	

HLA, Human Leukocyte Antigen; HCMV, Human cytomegalovirus; D, Donor; R, Recipient; D-, negative donor; R+, positive recipient

Patient 5

HCMV Monitoring

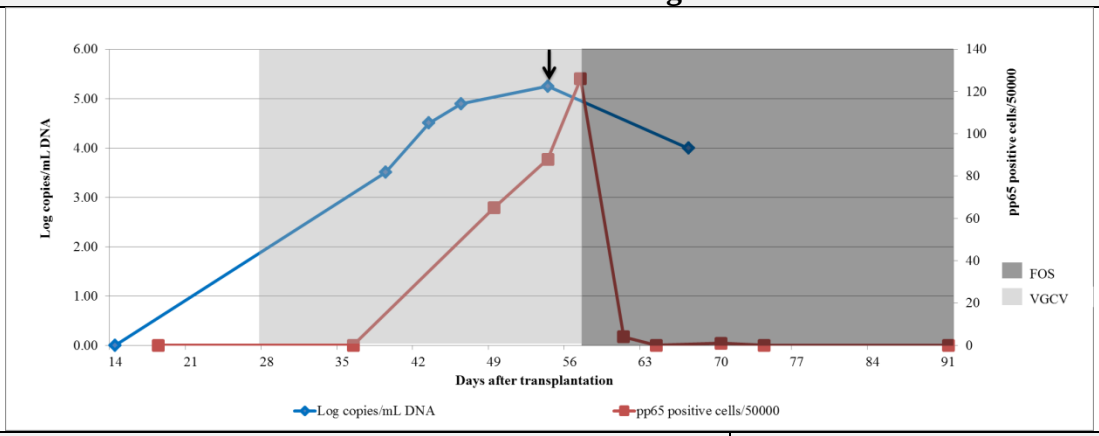


Characteristics of patient		Antiviral treatment
Gender	Male	**Day +28** – FOS 700 mg 8h/8h 24 days; **Day +53** – FOS 1176 mg 8h/8h 11 days; **Day +65** – VGCV 260 mg 2x/day 14 days.
Age (years old)	1	
Underlying disease	Primary immunodeficiency	
Conditioning regimen	Myeloblative	
Stem cell source	Cord Blood	
HLA status	Mismatch or Unrelated	
HCMV D/R status	D-/R+	

HLA, Human Leukocyte Antigen; HCMV, Human cytomegalovirus; D, Donor; R, Recipient; D-, Negative Donor; R+; positive recipient

Patient 6

HCMV Monitoring

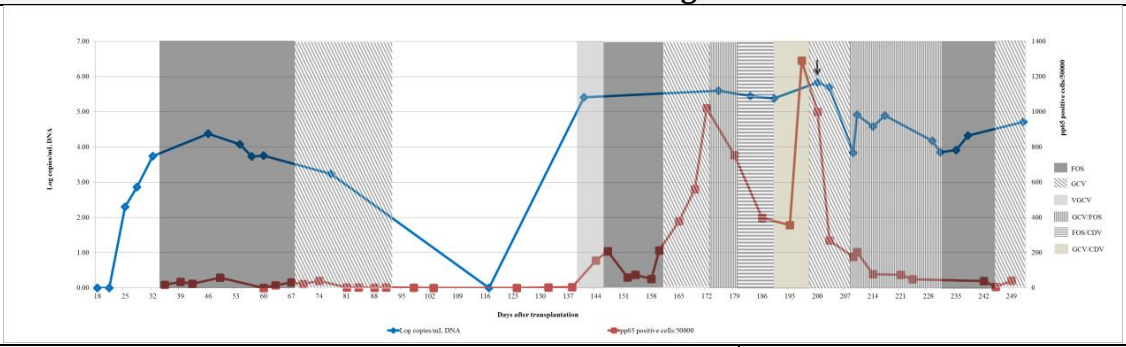


Characteristics of patient		Antiviral treatment
Gender	Male	**Day +28** – VGCV 450 mg 12h/12h 29 days; **Day +57** – FOS 1848 mg 8h/8h 32 days; **Day +75** – FOS 2808 mg 1x/day 16 days; **Day +91** – FOS 1224 mg 8h/8h.
Age (years old)	10	
Underlying disease	Acute myeloid leukemia	
Conditioning regimen	Myeloblative	
Stem cell source	Peripheral Blood	
HLA status	Mismatch or Unrelated	
HCMV D/R status	D+/R+	

HLA, Human Leukocyte Antigen; HCMV, Human Cytomegalovirus; D, Donor; R, Recipient; D+, positive donor; R+, positive recipient

Patient 7

HCMV Monitoring

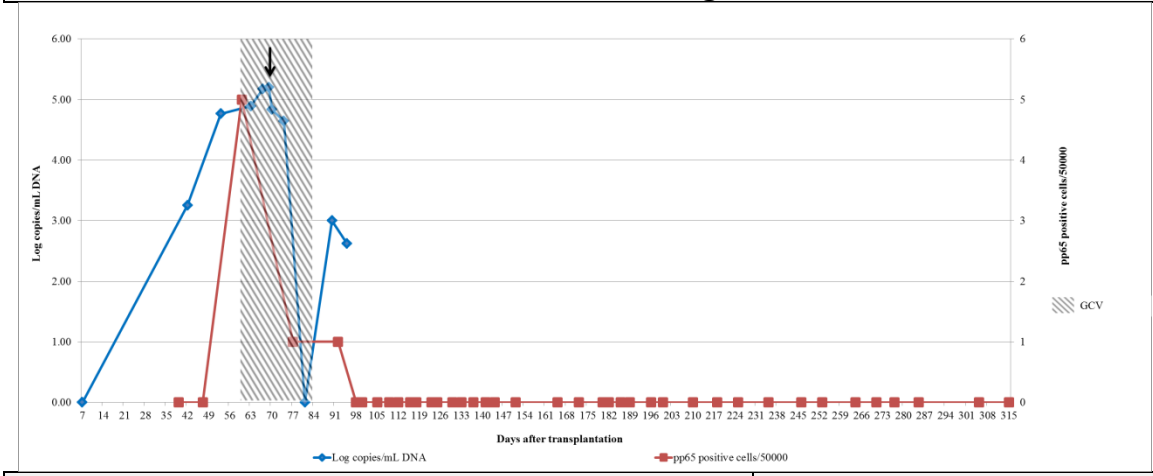


Characteristics of patient		Antiviral treatment
Gender	Female	**Day +34** – FOS 1200 mg 8h/8h 27 days; **Day +60** – FOS 1776 mg 2x/day 7 days; **Day +66** – GCV 98 mg 12h/12h 18 days; **Day +85** – GCV 45 mg 12h/12h 8 days; **Day +139** – VGCV 330 mg 12h/12h 6 days **Day +146** – FOS 920 mg 8h/8h 15 days; **Day +161** – GCV 89 mg 12h/12h 20 days; **Day +173** – FOS 900 mg 8h/8h 16 days; **Day +181** – CDV 16 mg 2x/day 17 days; **Day+189** – GCV 75 mg 12h/12h 40 days; **Day +204** – FOS 900 mg 8h/8h 38 days; **Day +245** – GCV 65 mg 1x/day 11 days.
Age (years old)	5	
Underlying disease	Acute lymphoid leukemia T lineage	
Conditioning regimen	Myeloblative	
Stem cell source	Cord Blood	
HLA status	Mismatch or Unrelated	
HCMV D/R status	D+/R+	

HLA, Human Leukocyte Antigen; HCMV, Human Cytomegalovirus; D, Donor; R, Recipient; D+, Positive Donor; R+, positive recipient

Patient 8

HCMV Monitoring

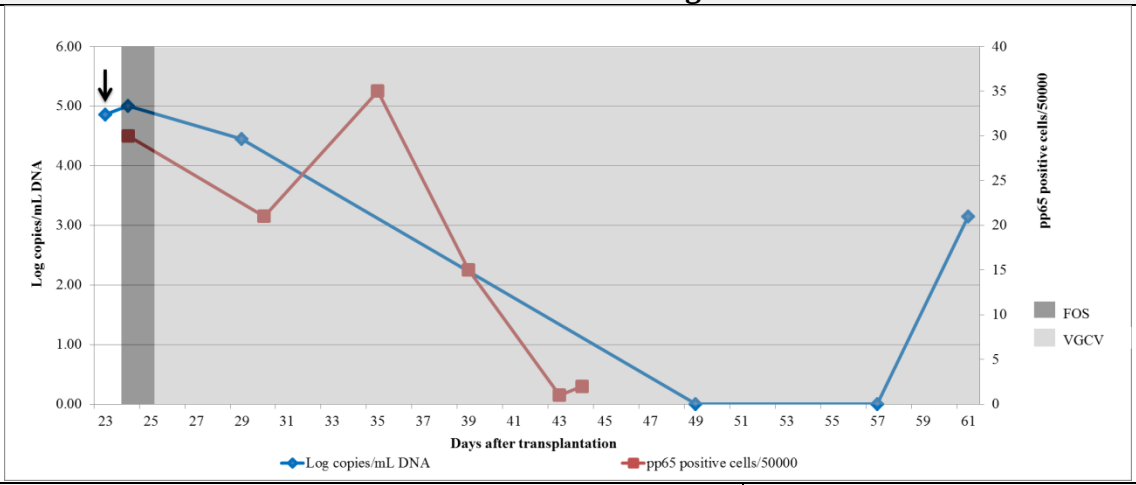


Characteristics of patient		Antiviral treatment
Gender	Male	**Day +61** – GCV 75 mg 12h/12h 22 days
Age (years old)	2	
Underlying disease	Medullar aplasia	
Conditioning regimen	Reduced intensity	
Stem cell source	Bone Marrow	
HLA status	Mismatch or Unrelated	
HCMV D/R status	D+/R+	

HLA, Human Leukocyte Antigen; HCMV, Human Cytomegalovirus; D, Donor; R, Recipient; D+, Positive Donor; R+, positive recipient

Patient 9

HCMV Monitoring

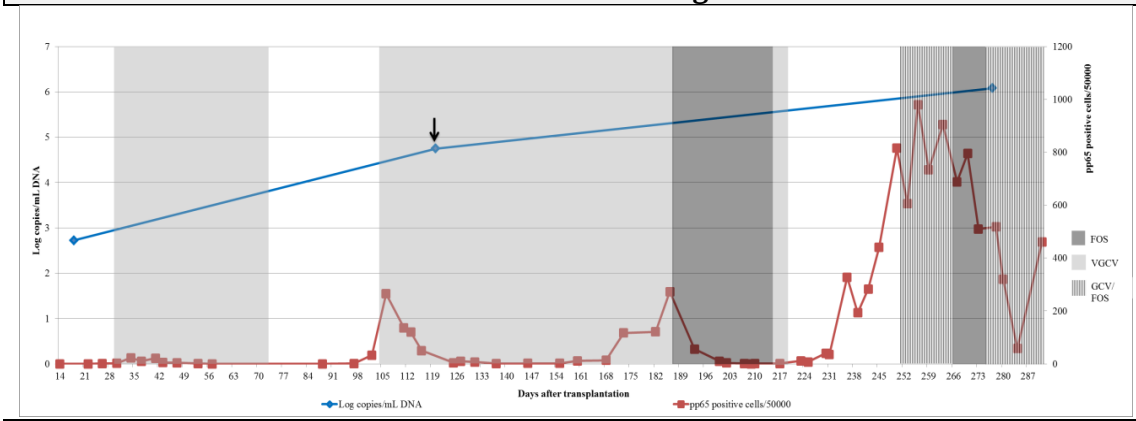


Characteristics of patient		Antiviral treatment
Gender	Male	**Day +24** – FOS 4728 mg 8h/8h 1 day; **Day +25** – VGCV 400 mg 12h/12h 38 days.
Age (years old)	60	
Underlying disease	Acute myeloid leukemia M1	
Conditioning regimen	Reduced intensity	
Stem cell source	Peripheral Blood	
HLA status	Mismatch or Unrelated	
HCMV D/R status	D+/R+	

HLA, Human Leukocyte Antigen; HCMV, Human Cytomegalovirus; D, Donor; R, Recipient; D+, Positive Donor; R+, positive recipient

Patient 10

HCMV Monitoring

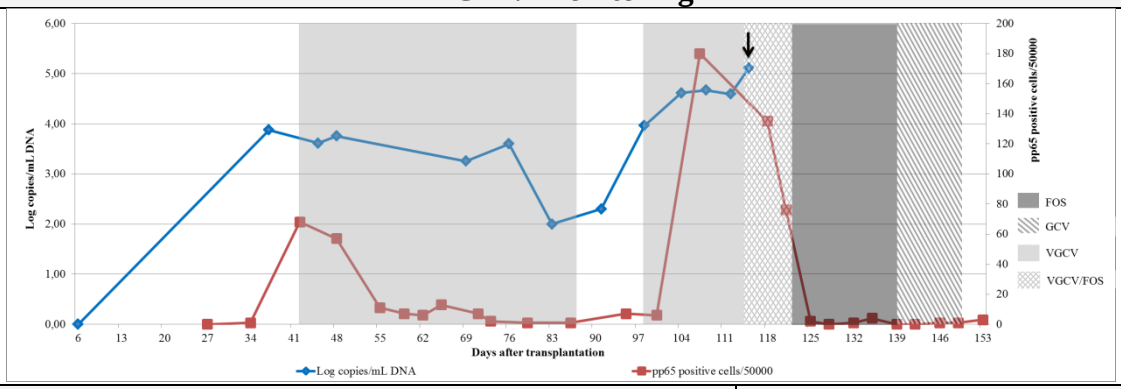


Characteristics of patient		Antiviral treatment
Gender	Female	**Day +30** – VGCV 900 mg 12h/12h 44 days; **Day +103** – VGCV 900 mg 12h/12h 13 days; **Day +120** – VGCV 900 mg 12h/12h 5 days; **Day +131** – VGCV 900 mg 12h/12h 55 days; **Day +187** – FOS 3288 mg 8h/8h 24 days; **Day +211** – VGCV 450 mg 12h/12h 6 days; **Day +251** – GCV 250 mg 12h/12h 16 days; **Day +252** – FOS 3288 mg 8h/8h 22 days; **Day +276** – GCV 280 mg 12h/12h 15 days; **Day +276** – FOS 3360 mg 8h/8h 15 days.
Age (years old)	49	
Underlying disease	Acute myeloid leukemia	
Conditioning regimen	Myeloblative	
Stem cell source	Peripheral Blood	
HLA status	Mismatch or Unrelated	
HCMV D/R status	D-/R+	

HLA, Human Leukocyte Antigen; HCMV, Human cytomegalovirus; D, Donor; R, Recipient; D-, negative donor; R+, positive recipient

Patient 11

HCMV Monitoring



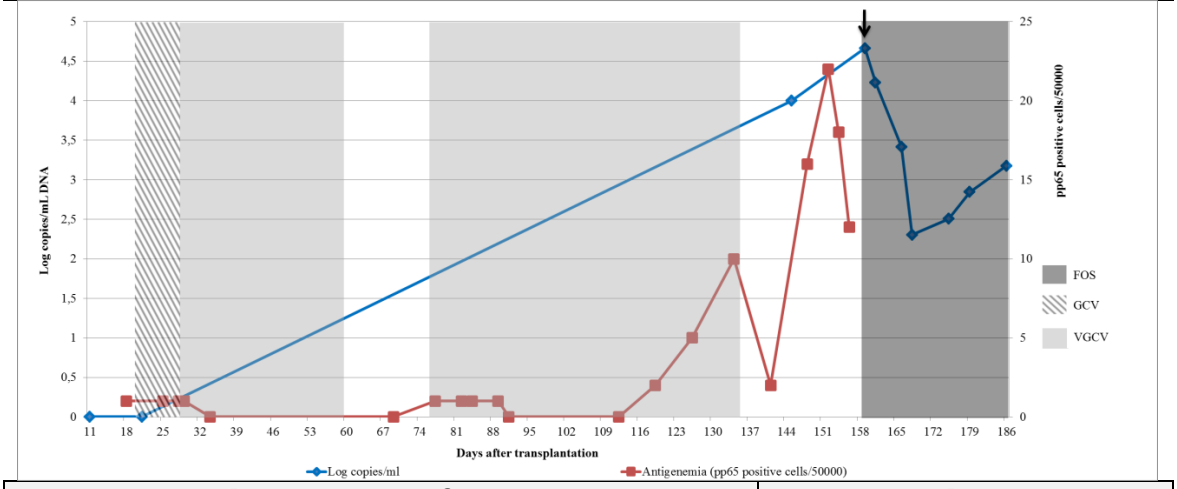
Characteristics of patient	
Gender	Female
Age (years old)	18
Underlying disease	Medullar aplasia
Conditioning regimen	Reduced Intensity
Stem cell source	Bone Marrow
HLA status	Mismatch or Unrelated
D/R HCMV status	D-/R+

Antiviral treatment
Day +42 – VGCV 900 mg 12h/12h 41 days
Day +84 – VGCV 450 mg 12h/12h 2 days
Day +98 – VGCV 900 mg 12h/12h 24 days
Day +115 – FOS 2640 mg 12h/12h 3 days
Day +119 – FOS 4080 mg 8h/8h 2 days
Day +122 – FOS 3360 mg 12h/12h 4 days
Day +123 – FOS 4080 mg 1x/day 8 days
Day +132 – FOS 2688 mg 12h/12h 1 day
Day +133 – FOS 4080 mg 1x/day 6 days
Day +139 – GCV 300 mg 1x/day 2 days
Day +142 – GCV 170 mg 1x/day 7 days

HLA, Human Leukocyte Antigen; HCMV, Human cytomegalovirus; D, Donor; R, Recipient; D-, negative donor; R+, positive recipient

Patient 12

HCMV Monitoring



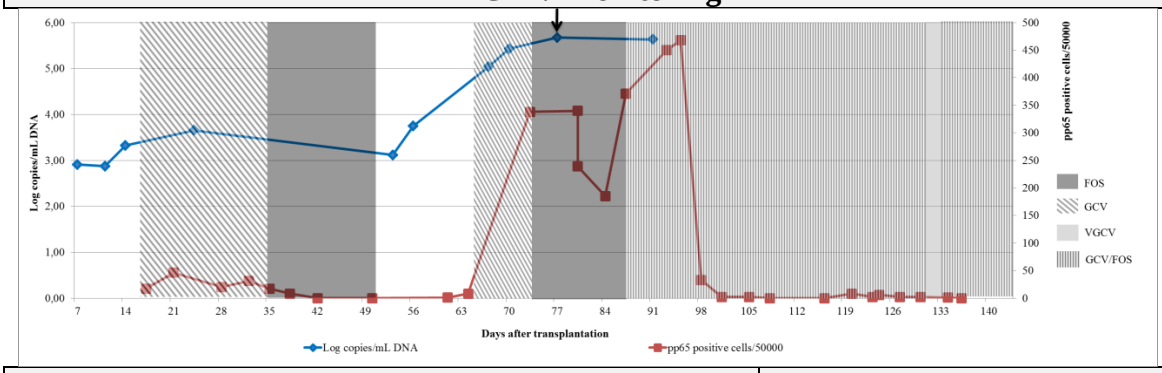
Characteristics of patient	
Gender	Male
Age (years old)	49
Underlying disease	Acute myeloid leukemia
Conditioning regimen	Myeloblative
Stem cell source	Peripheral Blood
HLA status	Mismatch or Unrelated
HCMV D/R status	D-/R+

Antiviral treatment
Day +19 – GCV 450 mg 12h/12h 6 days;
Day +28 – VGCV 900 mg 2 days;
Day +39 – VGCV 900 mg 4 days;
Day +54 – VGCV 450 mg 6 days;
Day +77 – VGCV 900 mg 57 days;
Day +159 – FOS 3600 mg 8h/8h 20 days;
Day +172 – FOS 3120 mg 12h/12h 15 days.

HLA, Human Leukocyte Antigen; HCMV, Human cytomegalovirus; D, Donor; R, Recipient; D-, negative donor; R+, positive recipient

Patient 13

HCMV Monitoring

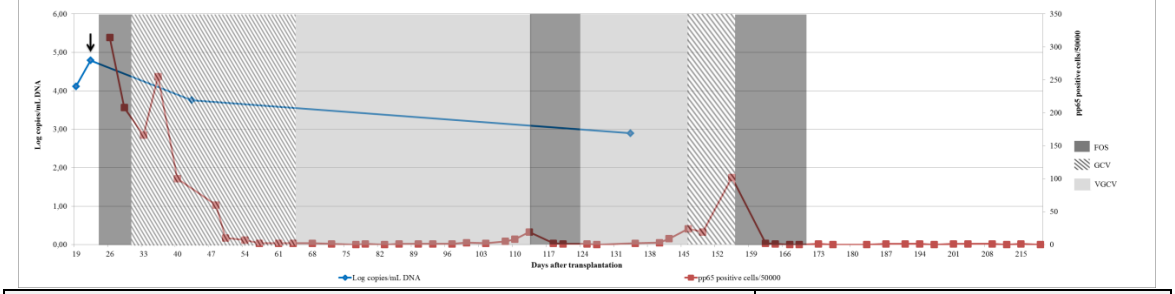


Characteristics of patient		Antiviral treatment
Gender	Female	**Day +17** – GCV 70 mg 12h/12h 18 days; **Day +35** – FOS 840 mg 8h/8h 12 days; **Day +47** – FOS 1200 mg 1x/day 3 days; **Day +67** – GCV 65 mg 12h/12h 7 days; **Day +74** – FOS 260 mg 8h/8h 39 days; **Day +89** – GCV 62,5 mg 12h/12h 32 days; **Day +114** – FOS 1125 mg 1x/day 7 days; **Day +121** – GCV 66 mg 12h/12h 9 days; **Day +121** - FOS 1200 mg 1x/day 9 days; **Day +130** – VGCV 240 mg 12h/12h 3 days; **Day +134** – FOS 840 mg 8h/8h 9 days; **Day +134** - GCV 70 mg 12h/12h 9 days.
Age (years old)	2	
Underlying disease	Acute myeloid leukemia M5	
Conditioning regimen	Myeloblative	
Stem cell source	Cord Blood	
HLA status	Mismatch or Unrelated	
HCMV D/R status	D-/R+	

HLA, Human Leukocyte Antigen; HCMV, Human cytomegalovirus; D, Donor; R, Recipient; D-, negative donor; R+, positive recipient

Patient 14

HCMV Monitoring

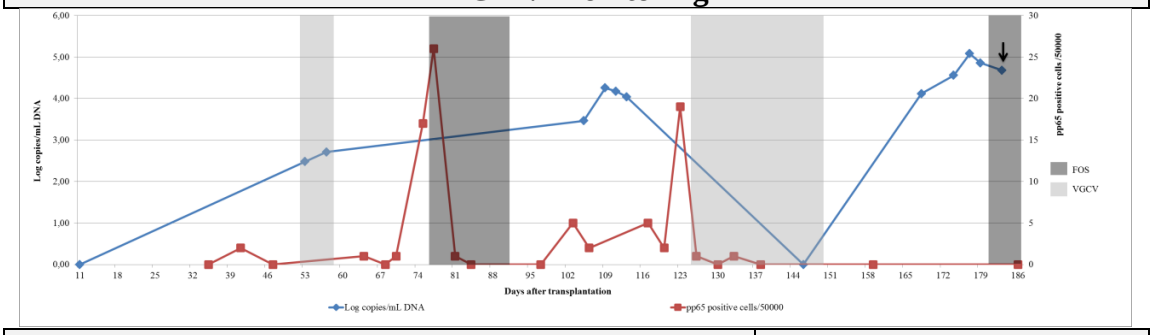


Characteristics of patient		Antiviral treatment
Gender	Female	**Day +23** – FOS 792mg 8h/8h 6 days; **Day +29** – GCV 75 mg12h/12h 35 days; **Day +64** – VGCV 360 mg 12h/12h 26 days; **Day +93** – VGCV 360 mg 12h/12h 4 days; **Day +100** – VGCV 360 mg 12h/12h 4 days; **Day +110** – VGCV 360 mg 12h/12h 3 days; **Day +113** – FOS 840 mg 8h/8h 7 days; **Day +120** – FOS 560 mg 8h/8h 1 day; **Day +126** – VGCV 100 mg 12/12h 8 days; **Day +134** – VGCV 150 mg 12h/12h 1 day; **Day +140** – VGCV 150 mg 12h/12h 2 days; **Day +146** – GCV 30 mg 12h/12h 9 days; **Day +155** – FOS 792 mg 8h/8h 15 days.
Age (years old)	2	
Underlying disease	Chronic Myeloid Leukemia	
Conditioning regimen	Myeloblative	
Stem cell source	Cord Blood	
HLA status	Mismatch or Unrelated	
HCMV D/R status	D-/R+	

HLA, Human Leukocyte Antigen; HCMV, Human cytomegalovirus; D, Donor; R, Recipient; D-, negative donor; R+, positive recipient

Patient 15

HCMV Monitoring



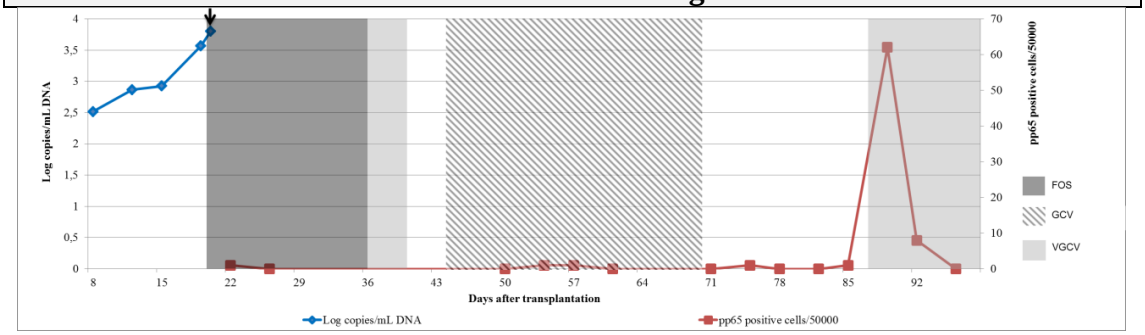
Characteristics of patient	
Gender	Male
Age (years old)	37
Underlying disease	Hodgkin's disease
Conditioning regimen	Reduced intensity
Stem cell source	Peripheral Blood
HLA status	Match/Related
HCMV D/R status	D+/R+

Antiviral treatment
Day +53 – VGCV 450 mg 2x/day 4 days; **Day +77** – FOS 4080 mg 8h/8h 5 days; **Day +82** – FOS 3048 mg 8h/8h 8 days; **Day +126** – VGCV 900 mg 12h/12h 21 days; **Day +182** – FOS 2640 mg 12h/12h 8 days

HLA, Human Leukocyte Antigen; HCMV, Human cytomegalovirus; D, Donor; R, Recipient; D+, positive donor; R+, positive recipient

Patient 16

HCMV Monitoring



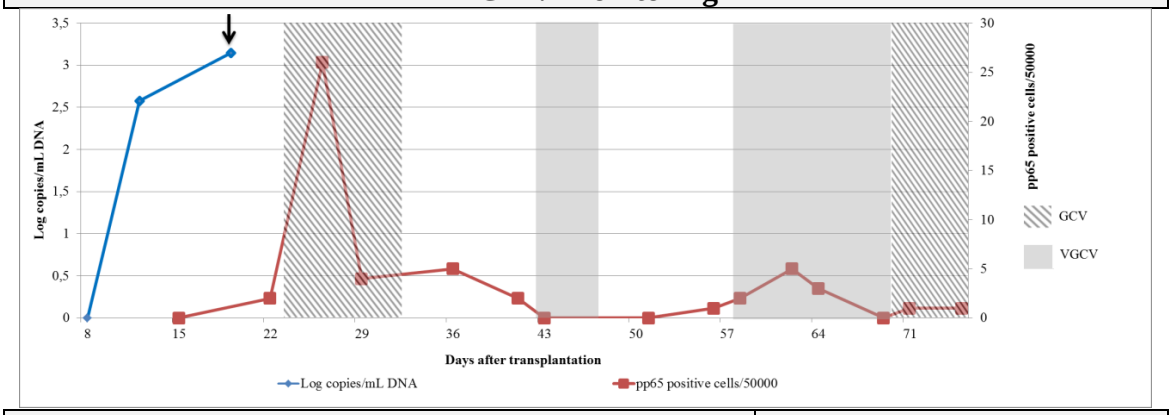
Characteristics of patient	
Gender	Male
Age (years old)	41
Underlying disease	Acute lymphoid leukemia
Conditioning regimen	Myeloblative
Stem cell source	Peripheral Blood
HLA status	Match/Related
HCMV D/R status	D-/R+

Antiviral treatment
Day +20 – FOS 3360 mg 8h/8h 1 day; **Day +22** – FOS 2688 mg 8h/8h 3 days; **Day + 26** – FOS 2472 mg 8h/8h 2 days; **Day + 29** – FOS 2400 mg 12h/12h 3 days; **Day +33** – FOS 2800 mg 1x/day 3 days; **Day +36** – VGCV 450 mg 1x/day 3 days; **Day +47** – GCV 35 mg 1x/day 7 days; **Day +55** – GCV 140 mg 1x/day 6 days; **Day +62** – GCV 65 mg 1x/day 2 days; **Day +65** – GCV 135 mg 1x/day 1 day; **Day +66** – GCV 67, 5 mg 1x/day 4 days **Day +89** – VGCV 450 mg 1x/day 9 days.

HLA, Human Leukocyte Antigen; HCMV, Human cytomegalovirus; D, Donor; R, Recipient; D-, negative donor; R+, positive recipient

Patient 17

HCMV Monitoring

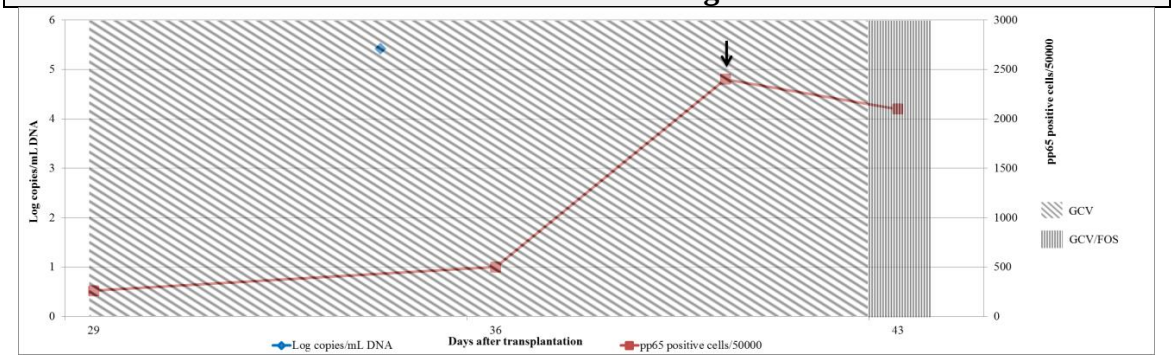


Characteristics of patient		Antiviral treatment
Gender	Female	**Day +23** – GCV 300 mg 12h/12h 8 days; **Day +43** – VGCV 900 mg 12h/12h 4 days; **Day +58** – VGCV 900 mg 12h/12h 12 days; **Day +70** – GCV 300 mg 12h/12h 5 days.
Age (years old)	23	
Underlying disease	Acute lymphoid leukemia	
Conditioning regimen	Myeloblative	
Stem cell source	Peripheral Blood	
HLA status	Mismatch or Unrelated	
HCMV D/R status	D+/R+	

HLA, Human Leukocyte Antigen; HCMV, Human cytomegalovirus; D, Donor, R, Recipient; D+, positive donor; R+, positive recipient

Patient 18

HCMV Monitoring

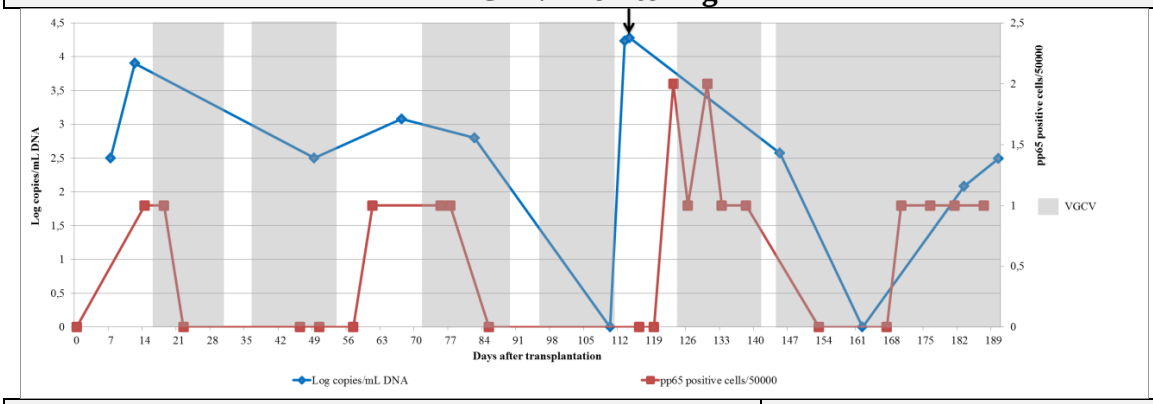


Characteristics of patient		Antiviral treatment
Gender	Male	**Day +29** – GCV 55 mg 12h/12h 15 days; **Day +42** – FOS 600 mg 8h/8h 2 days.
Age (years old)	1	
Underlying disease	Acute myeloid leukemia M0	
Conditioning regimen	Myeloblative	
Stem cell source	Cord Blood	
HLA status	Mismatch or Unrelated	
HCMV D/R status	D-/R+	

HLA, Human Leukocyte Antigen; HCMV, Human cytomegalovirus; D, Donor; R, Recipient; D-, negative donor; R+, positive recipient

Patient 19

HCMV Monitoring



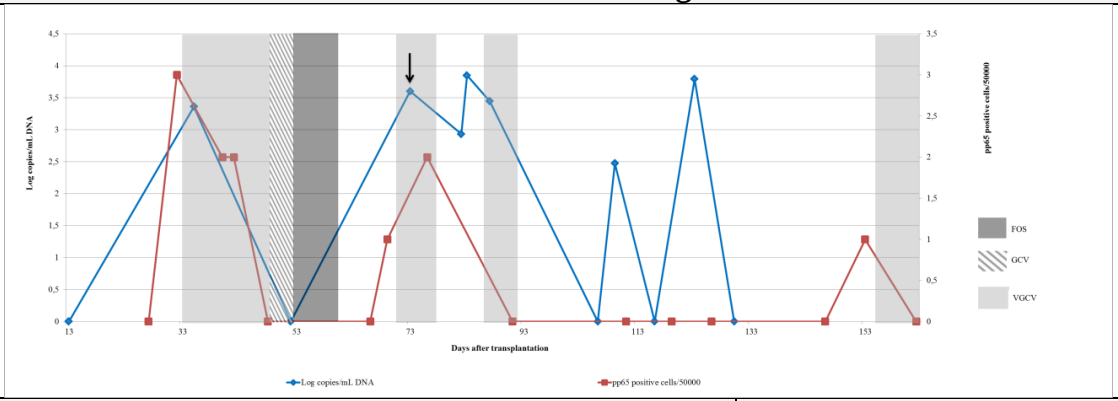
Characteristics of patient	
Gender	Female
Age (years old)	54
Underlying disease	Chronic Lymphoid Leukemia B lineage
Conditioning regimen	Reduced Intensity
Stem cell source	Peripheral Blood
HLA status	Match/Related
HCMV D/R status	D+/R+

Antiviral treatment
Day +15 – VGCV 900 mg 12h/12h 14 days; **Day +36** – VGCV 450 mg 1x/day 7 days; **Day +46** – VGCV 450 mg 1x/day 5 days; **Day +71** – VGCV 900 mg 1x/day 4 days; **Day +75** – VGCV 450 mg 1x/day 14 days; **Day +96** – VGCV 450 mg 1xday 14 days; **Day +124** – VGCV 450 mg 1x/day 17 days; **Day +145** – VGCV 400 mg 1x/day 45 days

HLA, Human Leukocyte Antigen; HCMV, Human cytomegalovirus; D, Donor; R, Recipient; D+, positive donor; R+, positive recipient

Patient 20

HCMV Monitoring



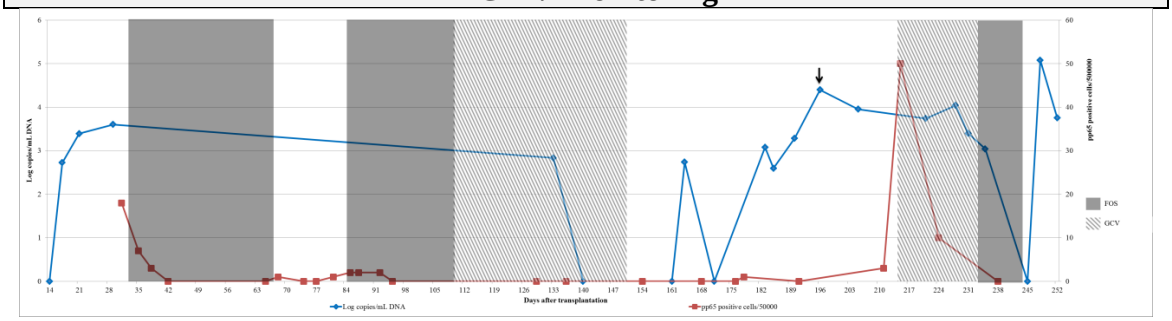
Characteristics of patient	
Gender	Male
Age (years old)	30
Underlying disease	Medullar Aplasia
Conditioning regimen	Reduced Intensity
Stem cell source	Bone Marrow
HLA status	Match/Related
HCMV D/R status	D+/R+

Antiviral treatment
Day +33 – VGCV 900 mg 12h/12h 15 days; **Day +48** – GCV 350 mg 12h/12h 4 days; **Day +52** – FOS 4560 mg 8h/8h 6 days; **Day +70** – VGCV 450 mg 12h/12h 6 days; **Day +87** – VGCV 450 mg 2x/day 4 days; **Day + 154** – VGCV 900 mg 1x/day 17 days.

HLA, Human Leukocyte Antigen; HCMV, Human cytomegalovirus; D, Donor; R, Recipient; D+, positive donor; R+, positive recipient

Patient 21

HCMV Monitoring

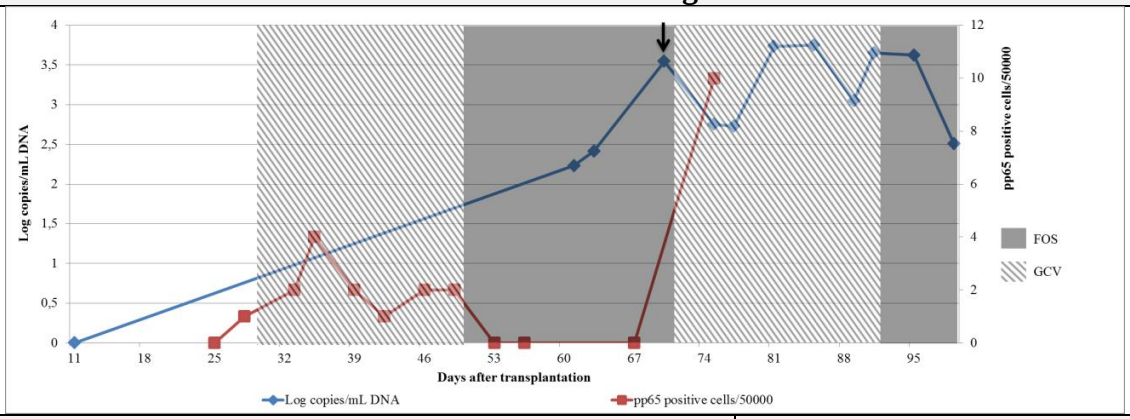


Characteristics of patient		Antiviral treatment
Gender	Female	**Day +32** – FOS 1080 mg 8h/8h 17 days; **Day +50** – FOS 1344 mg 8h/8h 16 days; **Day +85** – FOS 1121 mg 8h/8h 24 days; **Day +109** – GCV 100 mg 12h/12h 4 days; **Day +114** – GCV 50 mg 12h/12h 19 days; **Day +133** –GCV 50 mg 1x/day 16 days; **Day +215** – GCV 52.5 mg 12h/12h 6 days; **Day +221** – GCV 16 mg 1x/day 7 days; **Day +228** – GCV 50 mg 1x/day 4 days; **Day +232** – FOS 220 mg 8h/8h 9 days.
Age (years old)	6	
Underlying disease	myelodysplatic/myeloproliferative disease	
Conditioning regimen	Myelobaltive	
Stem cell source	Cord Blood	
HLA status	Mismatch or Unrelated	
HCMV D/R status	D+/R+	

HLA, Human Leukocyte Antigen; HCMV, Human Cytomegalovirus; D, Donor; R, Recipient; D+, positive donor; R+, positive recipient

Patient 22

HCMV Monitoring



Characteristics of patient		Antiviral treatment
Gender	Male	**Day +29** – GCV 300 mg 12h/12h 21 days; **Day +50** – FOS 3480 mg 8h/8h 7 days; **Day +58** – FOS 5184 mg 1x/day 13 days; **Day +71** – GCV 285 mg 2x/day 1 day; **Day +74** - GCV 140 mg 1x/day 17 days; **Day +92** – FOS 2400 mg 8h/8h 7 days.
Age (years old)	14	
Underlying disease	Acute Lymphoid Leukemia B(L3) lineage	
Conditioning regimen	Myeloblative	
Stem cell source	Peripheral Blood	
HLA status	Mismatch or Unrelated	
HCMV D/R status	D-/R+	

HLA, Human Leukocyte Antigen; HCMV, Human Cytomegalovirus; D, Donor; R, Recipient; D-, negative donor; R+, positive recipient

Attachment VI: Local ethical committee (CES IPO: 73/2015) approval

Parecer CES IPO: 73REAV/2015
Assunto: Estudo de Investigação ***Characterization of Cytomegalovirus resistant strains in hematopoietic stem cell transplanted patients.***
Investigadora: **Ana Bela Campos**
Data: 04 de Junho de 2015

PARECER

É parecer desta CES não existir impedimento de natureza ética ao desenvolvimento do referido estudo de investigação.

Dr. Artur Lima Bastos
Presidente da CES – IPO Porto EPE

Attachment VII: Review Article

TITLE:

HUMAN CYTOMEGALOVIRUS DRUG-RESISTANCE MUTATIONS IN STEM CELL TRANSPLANTATION: CURRENT STATE OF THE ART

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ABSTRACT

Human cytomegalovirus (HCMV) infection is a major cause of morbidity and mortality in allogeneic hematopoietic stem cell transplant (allo-HSCT) recipients. In these immunocompromised patients, the significant clinical impact of HCMV infection and progression to HCMV disease has been reduced by prophylactic and preemptive treatments using ganciclovir (GCV), valganciclovir (VGCV), foscarnet (FOS) and cidofovir (CDV). GCV/VGCV resistance results from mutations of phosphotransferase *UL97*, the viral DNA polymerase *UL54*, or both; whereas CDV and FOS resistance results from mutations of *UL54* only. This review will summarize the HCMV antiviral drug resistance, including the target genes and their functions, the mechanisms of antiviral resistance, the large number of mutations in *UL97* and *UL54* that have been identified in either clinical or laboratory isolates and the impact on HCMV susceptibility to drugs that it causes.

Keywords: Human cytomegalovirus, (aHSCT) recipients, ganciclovir, valganciclovir, foscarnet and cidofovir, phosphokinase (*UL97*), DNA polymerase (*UL54),* resistance.

Introduction

Human Cytomegalovirus (HCMV) alternatively known as Human Herpesvirus 5 (HHV-5) is a ubiquitous virus that belongs to the *Herpesviridae* family, *Betaherpesvirinae* subfamily [1, 2]. It is considered the prototype betaherpesvirus and shares with the other herpesviruses the virion structure, double-stranded DNA genome, kinetics of viral gene expression, persistence for the lifetime of the host after primary infection and reactivation from latency [3-9]. HCMV genome is the largest of all herpesviruses with approximately 230 000 base pairs (bp) containing a single origin of replication, a DNA polymerase gene (pUL54) and a complete package of genes required for viral DNA replication [10-14]. The AD169 laboratory strain was the first and the only completely sequenced HCMV strain and seems to have shorter genome than two laboratory strains (*Towne* and *Toledo*) and many clinical isolates [14, 15].

HCMV is a common virus and its epidemiology varies in different regions of the world and between socioeconomic and age groups [10, 16, 17]. Generally, the prevalence of HCMV infection is higher in developing countries and among persons of lower socioeconomic status [10, 18]. HCMV infection is in the majority of cases asymptomatic, since the virus is maintained in a state of latency or low level shedding that is clinically undetectable [8, 11, 19]. Nevertheless, HCMV infection is an important concern in certain risk groups and its reactivation from latency is critic, especially in immunocompromised patients, including HIV-positive individuals and transplant recipients [1, 5-7, 16, 18, 20].

CHAPTER I – HCMV infection and stem cell transplantation

HCMV infection is the leading viral cause of morbidity and mortality in patients who receive a solid organ transplant (SOT) (including kidney, liver, heart, heart-lung) or hematopoietic stem cell transplant (HSCT) [19, 21]. Several studies have described that HSCT recipients have a higher prevalence of HCMV infection and associated disease than SOT recipients [22, 23]. Moreover, it has been described that allogeneic-HSCT (allo-HSCT) recipients are at greater risk when compared to autologous recipients [14, 24]. Indeed, the incidence of HCMV infection after allo-HSCT ranges from 32% to 70%, varying with the serological status of the recipient and donor [14, 19, 25-29].

The risk of HCMV reactivation or disease in HSCT patients can be predicted prior the transplant, nevertheless some risk factors are dependent on the transplant and the outcome of the transplant [14, 24, 26, 27, 30-33] – Table I.

Typically, HCMV infection/reactivation appears within the first 100 days after transplant, both in allogeneic and autologous recipients, and affects mainly the lungs and the gastrointestinal tract [28, 30]. Furthermore, the increase of late-onset HCMV disease (occurring >100 days after transplant) may be due to antiviral prophylaxis or preemptive treatment during the first 100 days after transplantation [26, 31, 34-36] which inhibit the development of HCMV-specific T-cell lymphocyte response [24, 31, 37].

HCMV has both direct effects resulting from viral invasion of organ systems and indirect effects on the immune systems of recipients [19, 38]. The direct effects of HCMV primary infection or reactivation are the development of end-organ diseases such as pneumonia, hepatitis, pancreatitis, gastrointestinal disease, retinitis, encephalitis, colitis, esophageal ulcers and others [8, 14, 25, 38]. The indirect effects are frequently associated with increased risk of other infections, acute graft rejection and graft-*versus*-host disease (GVHD) [14, 19, 20, 38].

Table I: Conditions associated with the risk of developing of HCMV infection, reactivation, or disease in each of the following phase of therapy in HSCT patients. Adapted from [24].

BEFORE TRANSPLANT	AFTER TRANSPLANT
Host factors • Age • Underlying disease • Seropositivy status (donor and recipient) **Transplant-related factors** • T-cell depletion • Autologous vs allogeneic • Human Leukocyte Antigen (HLA) *(match vs non-match donors)* • Immunosuppression • Source of stem cells *(peripheral blood vs bone marrow)*	• Immunosuppression • Graft-versus-host disease (GVHD) • Immune reconstitution • Other viral infections • Opportunistic infections: (parasitic, bacterial and fungal)

CHAPTER II – HCMV infection management

Currently, three antiviral drugs have been shown to be effective in the prevention and/or treatment of HCMV infections and disease: ganciclovir (GCV), valganciclovir (VGCV), cidofovir (CDV) and foscarnet (FOS) [39-43]. Despite their clinical utility is limited by the efficacy, limited oral bioavailability, development of resistance in clinical practice, and associated toxicities, these drugs have been used to treat many forms of HCMV disease in immunocompromised patients [19, 44].

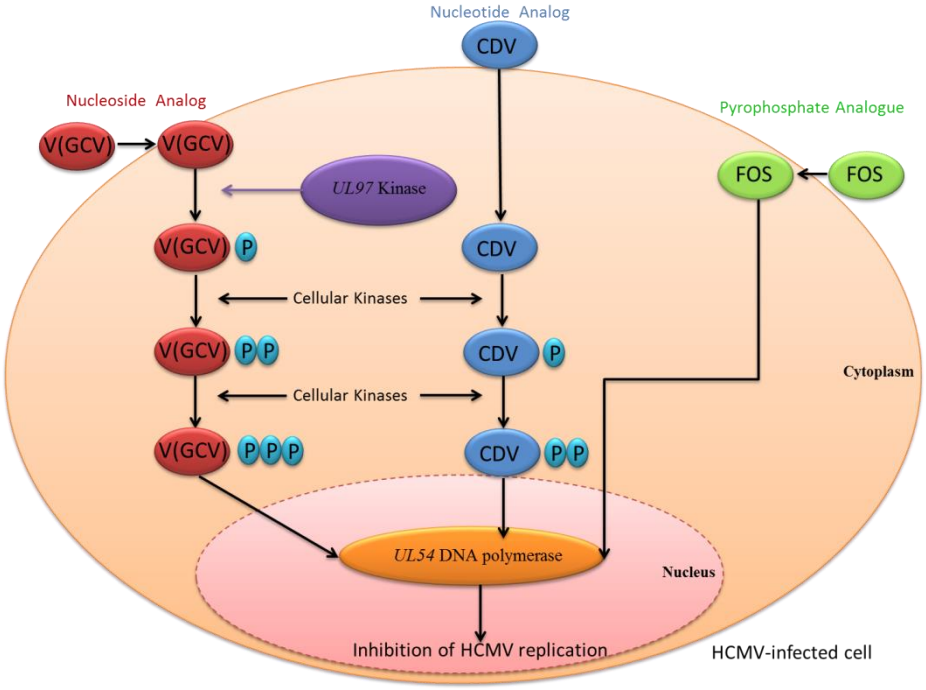

Figure 1: Action mechanisms of systemic antivirals approved for treatment of HCMV infections. GCV/VGC requires phosphorylation by the phosphokinase (pUL97). After monophosphorylation by pUL97, the cellular kinases add two additional phosphates. GCV triphosphate is the active form of the drug incorporated into viral DNA by the viral DNA polymerase (pUL54). CDV is a monophosphate analog and does not require initial viral phosphorylation. Cellular kinases add additional phosphates to produce CDV diphosphate, which is incorporated into the viral DNA by pUL54 leading to termination of viral DNA replication. FOS is a pyrophosphate analog, which does not require activation and is not incorporated into the growing viral DNA chain. It blocks directly the release of pyrophosphate by pUL54 and therefore resulting in chain termination. Adapted from [11, 45, 46].

Ganciclovir (GCV) and valganciclovir (VGCV)

Ganciclovir (GCV) is a nucleoside analog of 2´deoxyguanosine that act by inhibiting effective HCMV synthesis by a multistep process dependent on both viral and cellular enzymes [14, 19, 45]. The *UL97* gene of HCMV encodes a viral protein kinase (pUL97)

that phosphorylates GCV to GCV-monophosphate [5, 47-49] and two subsequent phosphorylation are performed by host cellular kinases that result in the formation of the GCV-triphosphate, which is a competitive inhibitor of the deoxyguanosine-triphosphate for the viral DNA polymerase encoded by *UL54* [14, 19, 39, 44, 45, 47, 50] – Figure 1.

GCV was initially approved by *Food and Drug Administration* (FDA) in 1989 for intravenous (IV) use [11, 19, 48]. GCV has been shown to reduce the severity of HCMV retinitis, gastrointestinal disease and, to a lesser extent, pneumonia in SOT, HSCT and *acquired immune deficiency syndrome* (AIDS) patients [14, 19, 25, 44]. Despite its high bioavailability and therapeutic efficacy, GCV use is limited due to the occurrence of hematologic side effects (neutropenia and thrombocytopenia) and it requires hospitalization for IV treatment [14, 19, 20, 24, 40, 51].

GCV poor oral bioavailability (5.6%) lead to the development of valganciclovir (VGCV), a L-valyl ester prodrug which after oral administration is rapidly metabolized in the liver and intestinal wall [11, 14, 19, 33, 44, 45, 48]. The adverse effects of VGCV are similar to those of GCV mainly, neutropenia and thrombocytopenia [14, 19, 33], nevertheless it has much better bioavailability (60%) and allows treatment without hospitalization [11, 14, 19, 33, 44, 45, 48]. Thus VGCV tends to be widely used among transplant recipients, not only for prophylaxis, but also for preemptive and maintenance therapy [19, 33, 40].
Although intravenous GCV and oral VGCV have been effective as first-line agents in both prevention and treatment of HCMV disease, the emergence of ganciclovir-resistant HCMV strains has posed a more significant threat due to an aggressive disease course and a greater mortality risk [39, 41, 51, 52].

Foscarnet (FOS)

Foscarnet (FOS) is an organic analog of inorganic pyrophosphate that reversibly and noncompetitively inhibits the activity of pUL54, the viral DNA polymerase [14, 44, 45, 47, 49, 50]. This noncompetitive inhibitor reversibly blocks the pyrophosphate binding site of pUL54 and inhibits the cleavage of pyrophosphate from deoxynucleoside triphosphates [19, 44] – Figure 1. FOS is administered as large volume IV solution since it must be present in high concentrations inside the cell to remain in contact with the viral DNA polymerase and inhibit DNA replication [5, 11, 14].

FOS was FDA approved in 1991 and despite its utility, it has been associated with nephrotoxicity and metabolic toxicity as well as renal failure, hypocalcemia, hypomagnesemia and hypophosphatemia [5, 11, 14, 19]. Due to its side effects, FOS is considered a second-line therapy, preferred over GCV especially in patients with myelosupression or weak graft after HSCT; to treat patients with AIDS and HCMV retinitis who are failing GCV therapy due to viral resistance; or those who cannot be treated with GCV due to dose-limiting neutropenia or leucopenia [14, 19, 20, 25, 51]. Furthermore, some studies refer that patients must be on long-term maintenance regimens with IV FOS to prevent the relapse or progression of HCMV disease [14, 25].

Cidofovir (CDV)

Cidofovir (CDV) is a monophosphate nucleotide analogue and does not require phosphorylation by pUL97 kinase, however it is dependent on diphosphorylation by cellular kinases converting it into CDV-diphosphate an analog of deoxycytosine [19, 44, 47, 49]. Similarly to GCV, the incorporation of CDV-diphosphate into viral DNA triggers a slowing and subsequent cessation of HCMV DNA replication [45, 47, 49] – Figure 1.

CDV was FDA approved in 1996 as an IV formulation for the treatment of a broad-range of DNA viruses infections, including all the herpesviruses [14, 44, 51, 53]. CDV oral bioavailability is less than 5% nevertheless it has a very long intracellular half-life when compared with GCV and FOS [11, 14, 19, 51]. CDV has excellent activity against HCMV and has been reported to be effective in the treatment of HCMV retinitis in AIDS patients and for HCMV infection and disease in allo-HSCT [14]. Despite its efficacy, there are some concerns regarding its poor oral bioavailability, dose-related nephrotoxicity and myelosuppression and therefore it is considered a third-line agent for HCMV infection [20, 51, 53].

CHAPTER III – HCMV drug-resistance analysis

Phenotypic Methods

There are important implications when virulent drug-resistant HCMV strains emerge in the clinical setting, including the need to developed laboratory methods for determining

susceptibilities of HCMV isolates to antiviral compounds [11, 17, 44, 46, 54, 55]. Phenotypic methods were the first to be developed before the establishment of genotypic testing, and they have been of great importance in identifying and characterizing mutations [11, 46]. These methods are based on determination of the drug concentration required to reduce viral growth [11, 45, 47, 56]. The "gold standard" method for phenotypic analysis of HCMV drug susceptibility has been the plaque reduction assay (PRA), where an inoculum of the clinical isolate is propagated in cell culture in the presence of different antiviral drug concentrations [44, 45]. Although the PRA assay is a simple test and directly assesses the IC50 (the effective dose in which a 50% plaque reduction is achieved) of a clinical isolate against specific antiviral agents, its standardization/reproducibility is still a problem, especially in context of definitions of cut-off levels for resistance [44-46, 56]. Other phenotypic methods have also been used for measuring HCMV drug susceptibility and resistance (ELISA tests, flow cytometry and DNA-DNA hybridization assays) [11, 45, 47, 56]. Moreover, efforts have been made to develop new assays (quantitative real-time PCR, reported cell lines, polymerase biochemical assays and marker transfer) more amenable to standardization and easier to perform [44, 46]. Although of the importance of phenotypic assays to diagnose antiviral drug resistance, they are subject to selection bias introduced during the growth of mixed viral, and lack sensitivity for the detection of low-level resistance or minor resistant subpopulations [44]. Phenotypic resistance assays are not commonly used to assess for resistant HCMV in clinical scenarios, however they remain essential for validation of genotypic assays [11].

Genotypic Methods

Genotypic assays were designed to detect mutations in *UL97* and *UL54* recognized to confer resistance to antiviral agents directly in clinical samples [45, 47]. The "gold standard" genotypic method for the detection of resistant HCMV strains is *Sanger Sequencing* which allow the identification of all nucleotide and amino acid substitutions [41]. However, this approach often fails for viral loads of <1,000 HCMV copies/ml of patient plasma, and it may fail in the detection of mutations that are present in <10 to 20% of the viral population [41]. There are other methods also used for the detection of drug resistance associated mutations (restriction fragment length polymorphisms and real time PCR assays), which have been used especially for UL97 gene [11, 46, 47]. Actually, next-

generation sequencing (NGS) has been developing and it may allow the identification of drug resistance at earlier stages, prior to the predominance of drug resistant viral populations [41, 54, 57-59]. Indeed, NGS enable the detection of smaller viral subpopulations (1-3%) with suitable precautions for errors introduced during amplification.

Genotypic analysis has proven to be fast and sensitive, providing results much sooner, minimizing selection bias by omitting the need to grow the virus and allowing the possibility to test directly on patient specimens and the [44, 60]. Nevertheless, its main disadvantage is that resistance mutations cannot be distinguished from sequence polymorphisms without prior confirmation by phenotypic analysis [45].

Recombinant Phenotyping Techniques

The discrimination between polymorphism and resistant-mutation is determined by recombinant phenotyping using marker transfer methodologies [55, 61]. Marker transfer has been developed to generate recombinant HCMV viruses and characterize the impact of specific genetic changes on viral susceptibility and resistance to antiviral drugs [60].

Although recombinant phenotyping has been technically difficult because of the large size of the HCMV genome, its efficiency has been considerably improved in recent years using cotransfection and restriction ligase techniques [60-62]. However, this method presents some difficulties such as: the natural rate recombination during HCMV replication is low (and therefore inefficient); is laboring; the risk of introducing new mutations after propagation under drug exposure; and substitutions associated with clinical drug resistance do not always confer obvious reductions in phenotypic susceptibility in cell culture or biochemical assays [11, 44, 60, 62]. More recently, bacterial artificial chromosome (BAC) technology has been applied to generate clonal recombinant viruses for marker transfer experiments and is now becoming the method of choice for the characterization of the many still uncharacterized resistance-associated substitutions [11, 44, 54, 61, 62].

CHAPTER IV – HCMV drug resistance

The emergence of HCMV resistance to one or more antiviral agents in immunocompromised patients is associated with treatment failure and progression of

HCMV disease [40, 41, 63, 64]. Overall, HCMV resistance ranges between 5.0-12.5% for SOT recipients and <4.0% for HSCT recipients [11, 41, 44, 48].

The rates of HCMV drug resistance vary widely dependent on type of patients, and several risk factors have already been identified: 1) patient and disease-related factors such as SOT, underlying disease, type and degree of host immunosuppression and the occurrence of HCMV disease [44, 54, 55, 65]; 2) treatment-related factors such as the prolonged antiviral therapy, suboptimal antiviral concentrations due to poor compliance or low drug absorption and limited oral bioavailability [19, 40, 41, 44, 51, 65-67]; and 3) viral factors such as the establishment of lifelong latency, which allows for late reactivations, the slow lytic replication cycle and higher viral load at the start of therapy [11, 40]. Indeed, drug resistance may be suspected if persistent or increasing plasma viral loads or overt HCMV disease occurs after two or more weeks of therapy [11, 41, 46, 51, 63, 65].

The resistance to HCMV antiviral drugs has been associated with single or multiple mutations in the viral phosphokinase (pUL97) and DNA polymerase (pUL54) that confer various levels of resistance [11, 40, 46, 47, 50, 56, 60, 65].

UL97 mutations

UL97 is a β2 delayed early gene located in the position 97 of the unique long region of HCMV genome and encodes for the viral phosphokinase pUL97 [11, 60, 62]. Structurally, pUL97 can be divided into different conserved functional regions: I, II, III, VIB, VII, VIII and IX [11, 44, 45, 54, 62] – Figure 2. While region I is responsible for adenosine triphosphate (ATP) binding, regions II, III, VIB and VII are involved in the phosphate transfer and region IX is essential for substrate binding [11, 46, 62].

pUL97 is required for phosphorylation of serine and threonine residues of various cellular and viral proteins, and is essential for efficient HCMV replication [11, 45, 54]. pUL97 has a crucial role in the phosphorylation of the GCV/VGCV, which is necessary for generation of its active forms and consequent inhibition of viral polymerase [44, 62]. Hence, mutations in *UL97* that impair this phosphorylation in virus-infected cells are the preferred mechanism of viral resistance [26, 37].

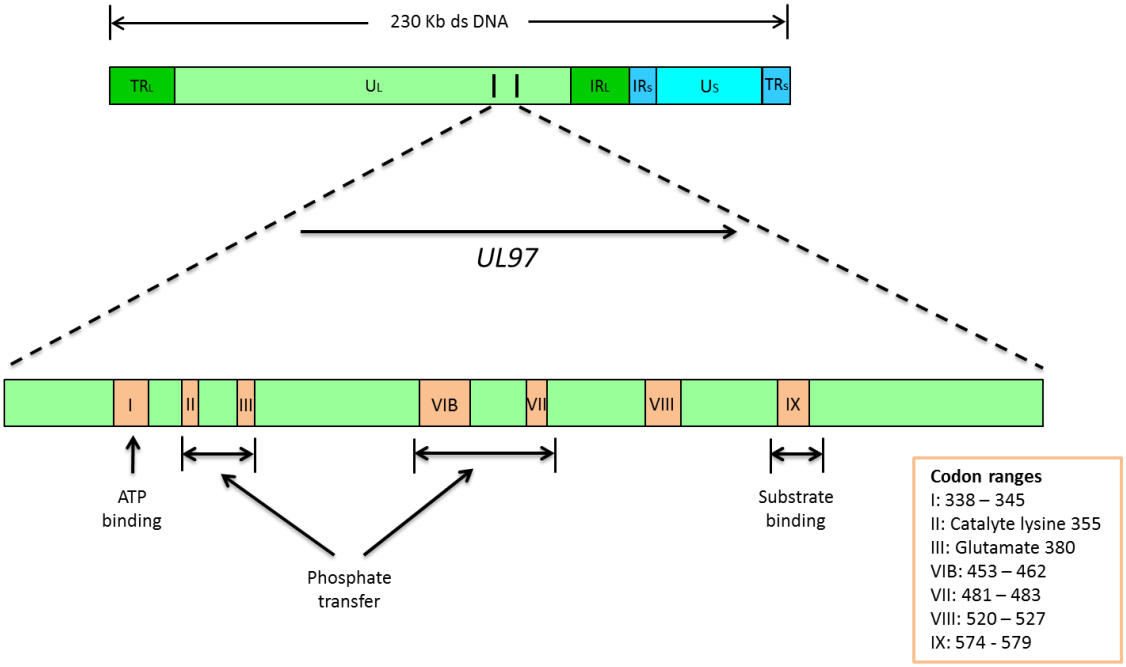

Figure 2: *UL97* structure and functional domains. Adapted from [11, 45, 46].

Approximately 95% of GCV/VGCV-resistant HCMV strains contain one or more mutations in *UL97* [26, 47, 49, 64, 68-70]. Typically, amino acid substitutions or short (1- to 17-amino-acid) in-frame deletions that change the ATP binding sites or phosphate transfer site alter the ability of pUL97 to phosphorylate GCV/VGCV [11, 42, 46, 54, 62, 68]. In *UL97*, known resistance-mutations are strongly clustered at codons 460 (region VIB) and 520 (region VIII), which are located at conserved kinase domains and therefore are essential for its function [11, 62]; and 590-607 (non functional region) which is dispensable for viral replication, nevertheless mutations in this region impair the recognition of GCV as a substrate [11, 62]. In fact, these regions encode a large variety of point mutations and deletions that confer different degrees of resistance to GCV/VGCV but do not affect susceptibility to FOS or CDV [11, 41, 44, 61, 62] – Table II.

By revising literature regarding the UL97 mutations in immunocompromised patients, including transplantation recipients and patients with AIDS, it was found that mutations in three specific codons (460, 594, and 595) accounting for approximately 70% of GCV-resistant HCMV strains [11, 44, 45, 49, 62, 68-70]. In fact, the most common *UL97* mutations associated with GCV resistance are M460I/V, H520Q, C592G, A594V, L595F/S/W, and C603W [45, 68-70]. All of these mutations have been described by recombinant phenotyping to confer a 5- to 16-fold increased GCV IC50 [45, 60-62, 68,

71], except C592G, which has been referred as preferentially selected in situations where the virus is exposed to low GCV concentrations [44, 45, 62, 68].

A variety of uncommon mutations (amino acid substitutions or deletions) that were reported to confer at least a 2-fold reduction in susceptibility to GCV are known to occur in different UL97 codons – Table II. Some of these mutations (L405P, V466G, A594E/P/T, L595T, E596G, I610T and A613V) confer low-grade resistance (2-4x increased IC50), while others (F342S, del355, V356G, D456N, M460T, C480R, C518Y, P521L, del590-600, del591-594, del591-607, C592F, A594G, del595, del595-603, E596Y, K599T, del601-603, C603R, C607Y/T, and del617) confer a moderate to higher GCV resistance (5-32x increased IC50) [46, 61, 68, 72-80]. Nevertheless, the del600, C603S, C607F, and E655K mutations have been shown to confer a <2-fold reduction in IC50 but have been reported to be clinically significant [11, 61, 68, 81].

There are also other mutations (M460L, del590-603, del594-595, del596, G598S, K599E, del601, T601M) that appear to confer significant GCV resistance based on recombinant phenotyping [82-87] and many other (V498I, A590T, A591D, E596D, N597I, G598V, K599M, del600-601, del601-602, C603Y, del597-603, C606D and C607S) that despite found in clinical isolates have not been yet phenotyped by marker transference [60, 61, 84]. Some of these non-phenotyped mutations were found in clinical isolates in combination with other mutations that confer resistance to GCV [40, 60, 61, 84, 88, 89].

Literature shows that there are several amino acid changes in *UL97* that do not correspond to resistant mutations, being considered as natural polymorphisms (e.g. H469Y, A478V, A588V, L600I, M615V, G623S, T659I and V665I) [61, 62, 90]. Nevertheless, there are some amino acid positions where single-base modifications change from natural polymorphism to resistant-mutation: K599R has been assessed as a polymorphism [61] while K599T was reported to induce GCV resistance [74]; and V466G, but not V466M, confers resistance to GCV [61, 90, 91]. Furthermore, some natural polymorphisms may modulate the drug-resistance level when combined with other mutations [67, 91]. Indeed, some authors suggest that the D605E could “partially or totally compensate” the effect of the GCV resistance, conferred by M460V [55, 62, 67] or A594P [55, 72]. Similarly, N510S mutation has been identified in combination with 591–594 deletion which is associated with resistance despite it has been shown that the level of resistance was not higher than the clinical isolate harboring the 591-594 deletion alone [68, 91].

Finally, there are some mutations described in *in vitro* studies (G340V, A442V, L446R, and F523C) that lead technically to GCV resistance, but are not expected to occur in clinical isolates since such mutations impair biological function by the loss of autophosphorylation and thus promote a severe growth deficiency [11].

Table II: Mutations in viral phosphokinase (*UL97*) gene associated with GCV/VGCV resistance in laboratory and clinical isolates confirmed by marked transfer.

Mutation	Amino acid change		Viral Phenotype GCV/VGCV (IC_{50})[a)]	References
	Wild type (Wt)	Mutant		
F342S	F	S	R (7.8)	[76]
del355	K	Del 1	R (16)	[76]
V356G	V	G	R (5.5)	[76]
L405P	L	P	R (2.5)	[61]
D456N	D	N	R (12)	[77]
M460I	**M**	**I**	**R (5)**	**[40, 65, 68, 75, 88, 92-97]**
M460L	M	L	NA	[50]
M460T	M	T	R (9.3)	[61, 65]
M460V	**M**	**V**	**R (8.3)**	**[41, 42, 50, 63, 65-68, 71, 75, 88, 90, 92, 94, 98-104]**
V466G	V	G	R (3.5)	[65, 90]
C480R	D	R	R (9)	[77]
C518Y	C	Y	R (12)	[105]
H520Q	**H**	**Q**	**R (10)**	**[41, 65, 68, 88, 98, 101, 102]**
P521L	P	L	R (17)	[76]
del591-594 [b)]	ACRA	Del 4	**R (6)**	[65, 68, 101] [106]
del590-600	AACRALENGKL	Del 11	R (6.3)	[78]
Del590-603	AACRALENGKLTHC	Del 14	NA	[107]
del591-607	ACRALENGKLTHCSDAC	Del 17	R (6.2)	[65, 68]
C592F	C	F	R (31.5)	[79]
C592G	**C**	**G**	**R (2.9)**	**[40, 41, 61, 63-65, 68, 71, 75, 90, 93, 98, 102-104] [108, 109]**
A594E	A	E	R (3.0)	[61, 65]
A594G	A	G	R (13.5)	[110]
A594P	A	P	R (2.9)	[40, 65, 72, 88, 103] [111]
A594T	A	T	R (2.7)	[27, 40, 64, 66, 68, 75, 88, 92, 98, 101] [112]
A594V	**A**	**V**	**R (8.3)**	**[40, 41, 50, 52, 57, 58, 61, 64-66, 68, 71, 75, 88, 92, 94, 95, 97, 98, 100-104, 111, 112]**
del594-595	AL	Del 2	NA	[85]
L595F	**L**	**F**	**R (15.7)**	**[65, 68, 75, 90, 94, 103] [113]**
L595S	**L**	**S**	**R (9.2)**	**[40-42, 52, 64-66, 68, 71, 75, 88, 92-95, 97, 98, 100-103, 112]**
L595T	L	T	R (2.2)	[75]
L595W	**L**	**W**	**R (5.1)**	**[52, 65, 68, 88, 102, 105]**
del595	L	Del	R (13.3)	[65, 102] [114]

del595-603	LENGKLTHC	Del 9	R (8.4)	[65, 73, 115]
del596	E	Del 1	NA	[86]
E596G	E	G	R (2.3)	[40, 65, 68]
E596Y	E	Y	R (6.4)	[46]
G598S	G	S	NA	[65, 82] [116]
K599E	K	E	NA	[86]
K599T	K	T	R (5.3)	[27, 74]
del600	L	Del	r (1.9	[65, 68, 102]
del601	T	Del	NA	[83, 102]
del601-603	THC	Del 3	R (11)	[65, 99]
T601M	T	M	NA	[87]
C603R	C	R	R (3.6 - 8.3)	[61, 65, 88, 90]
C603S	C	S	r (1.9)	[61, 65, 88]
C603W	**C**	**W**	**R (8)**	**[40, 41, 61, 65, 66, 68, 75, 88, 93, 97, 101-103, 109]**
C607F	C	F	r (1.9)	[41, 65, 68, 101]
C607T	C	T	R (12.5)	[80]
C607Y	C	Y	R (12.5)	[58, 65, 68, 88, 96, 101]
I610T	I	T	R (2.6)	[46]
A613V	A	V	R (2.3)	[117]
del617	Y	Del 1	R (10)	[77]
E655K	E	K	r (1.7)	[81]

Boldface indicates the most common mutations conferring resistance; Deletions start at the designated codon and continue through the number of shown codons; R, resistant strain (≥2 fold reduction in susceptibility to GCV/VGCV); r, low-grade resistance or <2 fold reduction in susceptibility to GCV/VGCV; NA, not available.

a) The level of resistance of each mutation is expressed as the ratio of the half maximal inhibitory concentration (IC_{50}) of the mutant to that of drug – sensitive wild-type (IC_{50} of mutant/IC_{50} of wild type ratio); b) Deletion of codons 591 to 594 results in the same mutant virus as deletion of codons 590 to 593;

UL54 mutations

The *UL54* gene is a β2 delayed early gene located on the position 54 of the unique long region of HCMV genome which encodes for the viral DNA polymerase (pUL54) [60]. Both DNA and amino acid sequence analysis revealed that this protein has significant homology to the *pol* genes of other herpesviruses but also with DNA polymerases encoded by a wide range of organisms [49, 60]. There are two functions extremely important in viral polymerases: the 3´-5´ exonuclease activity (Exo I through III) and polymerization (I through VII). In addition, the Delta-region (∂-region) C has also been suggested to participate in 3´-5´ exonuclease activity of pUL54 [11, 45] and there is an overlap of Exo II with polymerization region IV and of Exo III with ∂-region C [11, 45] – Figure 3.

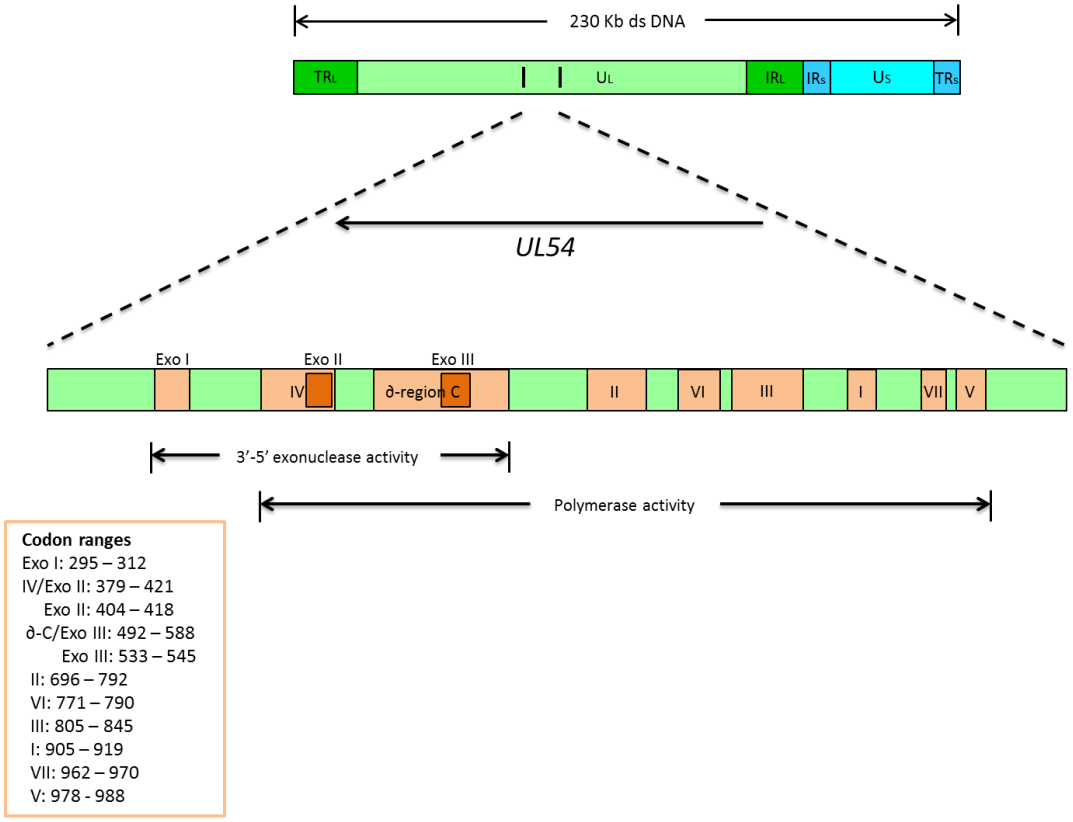

Figure 3: *UL54* structure and functional domains. Adapted from [11, 45, 46].

pUL54 is the central enzyme involved in viral DNA replication and the primary drug target for current therapies (GCV/VGCV, FOS and CDV) [42, 43, 60]. The mechanisms of resistance postulated for either GCV-triphosphate or CDV-diphosphate are: 1) decreased

affinity of the enzyme for the inhibitor; 2) decreased selective incorporation of the inhibitor into the elongating DNA chain; or 3) enhanced selective excision from the DNA chain of the incorporated inhibitors [11, 45]. Furthermore, as FOS is not incorporated into the DNA the resistance if often associated with decreased affinity of the enzyme [11, 45].

Mutations in *UL54*, despite less common than mutations in the *UL97*, are clustered over a much larger area of the gene and the positions of known resistance-conferring mutations are more varied [44, 50, 57]. Although uncommon, it has been reported the appearance of a resistance *UL54* mutations in the absence of a UL97 mutation [11, 47]. Indeed, the combination of amino acid substitutions in pUL97 and pUL54 resulted in greater reduction in susceptibility to GCV than in isolates with only one amino acid substitution [58, 71, 108].

As the majority of *UL54* resistant-mutations occur within the conserved regions of homology the particular resistance phenotype largely correlates with its location within the genome [42, 44, 45, 47] – Table III. Mutations that confer resistance to GCV and CDV are most often found within the exonuclease domains and region V, whereas mutations located within the C-terminal extremity of ∂-region C and within or next to conserved regions II and VI, seem to be mostly involved in FOS resistance [44, 45, 47, 54]. Deletion of codons 981-982 in region V causes resistance to GCV, CDV and FOS, while mutations within conserved region III can be associated with resistance to any single agent or combination of agents, and no drug resistance mutations have so far been detected in regions I and VII [11, 44, 45, 47].

Despite of the majority of UL54 mutations that confer resistance to GCV/VGCV also confer it to CDV and/or FOS [11, 50, 57, 65, 66] are there others that confer only to GCV or CDV or FOS [54, 97, 118]. The D542E and K805Q is the single mutation which confers resistance only to CDV [65, 75, 119, 120]; while L802V, P829S, L862F and L957F are the mutations which confers resistance only to GCV [54, 97, 118, 121]; and N495K, S585A, D588E, F595I, A692S, T700A, V715M, E756D/Q, W780V, T838A, M844T and V946L confer resistance only to FOS [15, 42, 54, 58, 65, 75, 95, 102-104, 118, 122, 123].

As in UL97, the impact on drug resistance of each mutation in UL54 is variable and has been demonstrated by several recombinant phenotyping studies – Table III. There are several mutations (Q578H, E756K, L773V, T813S, A834P, G841A and del 981–982) that

confer a ≥2-fold reduction for all three drugs, with A834P and Q578H to be the most frequently found [41, 50, 57, 58, 65, 103, 104, 118, 124]. Nevertheless, some (T821I and M844V) do not confer a ≥2-fold reduction to all of three drugs [11, 46, 60, 108, 113, 118, 119, 122, 124-126]. Cross-resistance between GCV and CDV has been reported more frequently than between the GCV and FOS [11, 46, 47, 56, 60, 65] and the most frequent mutations are P522S, A987G, L545S and T503I [40, 41, 50, 58, 65, 75, 88, 102, 103, 127, 128]. Among mutations that confer ≥2-fold resistance to GCV and CDV (D301N, N408D/K/S, N410K, F412C/L/S/V, D413A/E/N, P488R, K500N, L501I, T503I, K513E/N/R, L516R, I521T, P522A/S, V526L, C539G/R, L545S/W, V812L, A987G and del524) the majority to exhibit greater resistance to CDV than to GCV [11, 46, 60, 96, 118, 119, 122, 124, 125, 129]. Similarly, there are several mutations with cross-resistance (≥2-fold reduction) to GCV and FOS (D515E/Y, D588N, V776M, V781I, V787A/L, L802M, A809V and G841S), being the most frequent D588N, V781I, L802M and A809V, and with the majority to confer greater resistance to FOS than to GCV [46, 58, 65, 75, 88, 102, 103, 108, 109, 119-121, 124, 128, 130].

The mutations that confer higher rate of GCV-resistance are D413A, L501I, K513N and deletion of codons 981 to 982 which have been associated with a 6- to 8-fold increased resistance [11, 46, 60, 71, 113, 119, 122]; while for CDV are N408K, F412C/S/V, D413A/N, K513N/R, C539R, D542E and A987G which have been associated with a 10- to 21-fold decrease in susceptibility [11, 118, 119, 124, 125]; and for FOS are V715M, E756K, L802M, A809V, T821I and A834P which confer 5.5- to 21-fold increased resistance [108, 118-120, 122, 124, 125]. In addition, there are several mutations (A505V, T552N, Q578L, I726T/V, W780V, L802V, T821I, M844V and L862F) that confer a <2-fold reduction in susceptibility to HCMV-antiviral drugs in phenotypic assays and have been found in both laboratory and clinical isolates and lack more evidences [54, 58, 60, 118, 126, 130].

Despite of some UL54 mutations have little direct impact on HCMV susceptibility to drugs; they may augment the effect of other mutations, either by directly enhancing resistance or indirectly by increasing viral fitness [54, 60, 118, 120, 125]. For example, K805Q seem to improve the viral fitness (replication kinetics) of T821I which is associated with high resistance to FOS [120]. N408K in combination with A834P also partially reconstituted the replication impairment of recombinant virus containing only A834P

[125]. This suggests that perturbation of both polymerization (A834P) and exonuclease (N408K) activities contributes to increased GCV and CDV resistance ([125]). Others combinations, such as N408D/L957F, L545S/P829S and L957F/T552N showed FOS resistance whereas the single mutations did not [54, 118]. Moreover, some studies suggested that the occurrence of mutations within conserved regions contribute to FOS resistance due since it is associated with a slower replication of HCMV [95, 104, 123, 131].

Two polymorphisms laying in conserved regions (E506K in ∂-Region C and R785S in region VI) thought to be associated with resistance are described in a position with high variability amongst herpesviruses DNA polymerase, supporting the theory that they may be natural variants [127]. Nevertheless, polymorphisms in UL54 occur frequently outside the conserved regions and are relatively common which makes more complicated to distinguish them from true resistance mutations [11, 47]. For example, the P522L polymorphism, located at the same position as confirmed resistance-associated mutation (P522S), and the E315D, D879G and A972V polymorphisms have also been observed in patients failing treatment or prophylaxis [40, 60]. Furthermore, there are several other amino acid substitution (i.e. M393R/K, L501F, L516M, P608S, T610M, A614S, G629S, I722V, Y751H, S880L, S897P and R1052C) found in clinical isolates that have not been phenotyped by marker transfer yet [40, 56, 75, 97, 103, 132]. A number of other mutations observed after *in vitro* or *in vivo* drug exposure have not been confirmed, and they illustrate the potential diversity and distribution of pol mutations, which may not be the same in laboratory strains as in clinical isolates [11].

Table III: Mutations in DNA polymerase (*UL54*) gene associated with antiviral resistance in laboratory and clinical isolates confirmed by marked transfer.

Mutation	Amino acid change		Viral Phenotype [a)]			References
	Wild type (Wt)	Mutant	GCV (IC_{50})	CDV (IC_{50})	FOS (IC_{50})	
Exo I (295 - 312)						
D301N	D	N	**R (2.6)**	**R (3)**	S (0.5)	[65, 122]
IV / Exo II (379 - 421 / 404 - 418)						
N408D	N	D	**R (4.9)**	**R (5.6)**	S (1.3)	[50, 58, 65, 75, 98, 103, 118, 119, 125]
N408K	N	K	**R (4.2)**	**R (21)**	S (0.7)	[50, 65, 103, 125]
N408S	N	S	**R (3.1)**	**R (7.5)**	S (1.0)	[96]
N410K	N	K	**R (2.9)**	**R (3)**	S (0.8)	[65, 122]
F412C	F	C	**R (4.2)**	**R (18)**	S (1.2)	[41, 65, 75, 93, 109]
F412L	F	L	**R (4.6)**	**R (9.4)**	S (1.1)	[54, 65, 124]
F412S	F	S	**R (5.3)**	**R (13)**	S (0.8)	[54, 65, 88, 124]
F412V	F	V	**R (4.3)**	**R (15.5)**	S (1.1)	[65, 119]
D413A	D	A	**R (6.5)**	**R (11)**	S (0.8)	[65, 99]
D413E	D	E	**R (4.8)**	**R (4.3)**	S (0.8)	[65, 98, 103, 122]
D413N	D	N	**R (3.8)**	**R (10)**	S (1.0)	[58]
Outside conserved region						
P488R	P	R	**R (3.5)**	**R (7.9)**	S (0.6)	[54, 118]
∂-Region C (492 - 588)						
N495K	N	K	S (1.1)	S (1.1)	**R (3.4)**	[42, 65, 123]
K500N	K	N	**R (3.2)**	**R (3.0)**	S (1.2)	[54, 118]
L501I	L	I	**R (6)**	**R (9.1)**	S (1.4)	[41, 50, 65, 75, 119]
T503I	T	I	**R (2.9)**	**R (6.1)**	S (0.5)	[41, 65, 75, 122]
A505V	A	V	**r (1.8)**	**R (2)**	S (1.1)	[130]
K513E	K	E	**R (5)**	**R (9.1)**	S (1.4)	[65, 75, 93, 119]
K513N	K	N	**R (6)**	**R (12.5)**	S (1.5)	[65, 131]
K513R	K	R	**R (3.7)**	**R (10)**	S (1.1)	[58, 75]
D515E	D	E	**R (2.7)**	NT	**R (4.6)**	[103]
D515Y	D	Y	**R (5.6)**	NT	**R (4.6)**	[103]
L516R	L	R	**R (2.1)**	**R (5.1)**	S (0.8)	[65, 122]
I521T	I	T	**R (2.1)**	**R (5.1)**	S (0.8)	[65, 97, 129]
P522A	P	A	**R (3)**	**R (4.1)**	S (1)	[41, 65, 98, 129]
P522S	P	S	**R (3.1)**	**R (3.6)**	S (1.1)	[41, 42, 58, 65, 66, 88, 102, 119, 128]
del524	C	del1	**R (3.5)**	**R (9.7)**	S (1.1)	[96]
V526L	V	L	**R (5.5)**	**R (2.5)**	S 1.8)	[81]
ExoIII / ∂ region C (533 - 545 / 492 - 588)						
C539G	C	G	**R (3.1)**	**R (4.4)**	S (1.0)	[58]
C539R	C	R	**R (3.2)**	**R (13.3)**	S (0.7)	[54, 118]
D542E	D	E	S (1.5)	**R (12)**	S (1.7)	[133]
L545S	L	S	**R (3.5)**	**R (9.1)**	S (1.2)	[40, 65, 118, 119, 127]
L545W	L	W	**R (4.9)**	**R (6.3)**	S (1.3)	[54, 65, 124]
∂-Region C (492 – 588)						
T552N	T	N	**r (1.9)**	S (1.2)	**R (2.6)**	[54, 118]

Q578H	Q	H	**R (3.3)**	**R (2.3)**	**R (4.5)**	[54, 57, 58, 65, 124] [134]
Q578L	Q	L	**r (1.9)**	S (0.8)	**R (3.0)**	[58, 60]
S585A	S	A	S (1.5)	S (1.4)	**R (2.7)**	[54, 118]
D588E	D	E	S (1.3)	S (1.1)	**R (2.3)**	[65, 75, 119]
D588N	D	N	**R (3.8)**	**R (2.7)**	**R (3.2-9)**	[41, 65, 93, 104, 124]
Outside conserved region						
F595I	F	I	S (1.3)	S (1.2)	**R (2.0)**	[54, 118]
A692S	A	S	S (1.6)	S (1.7)	**R (3.3)**	[102, 122]
Region II (696-742)						
T700A	T	A	S (0.9)	S (1.5)	**R (4.7)**	[63, 65, 95, 98, 119]
V715M	V	M	S (1.0)	S (1.1)	**R (5.5)**	[58, 64, 65, 88, 95, 111, 119, 128]
I726T	I	T	**R (2.0)**	**r (1.7)**	S (1.1)	[130]
I726V	I	V	**r (1.9)**	**r (1.9)**	S (1.2)	[130]
Outside conserved region						
E756D	E	D	S (1.2)	S (0.7)	**R (3.4)**	[65, 98, 122]
E756K	E	K	**R (2.5)**	**R (2.2)**	**R (>8)**	[93, 122]
E756Q	E	Q	S (1.7)	S (1.0)	**R (4.3)**	[128]
Region VI (771- 790)						
L773V	L	V	**R (3.0)**	**R (2.5)**	**R (4.4)**	[58]
L776M	L	M	**R (2.5)**	S (1)	**R (3.5)**	[65]
W780V	W	V	S (1.5)	NA	**r (1.9)**	[126]
V781I	V	I	**R (1-4)**	S (1.2)	**R (4-5.2)**	[58, 75, 93, 102, 119, 128]
V787A	V	A	**R (2.5)**	NA	**R (3.5)**	[111]
V787L	V	L	**R (2.4)**	S (1)	**R (4.1)**	[102, 118, 128]
Outside conserved region						
L802M	L	M	**R (1.1-3.5)**	S (0.9-1.8)	**R (3.2-11)**	[41, 75, 93, 97, 103, 104, 109, 118, 119]
L802V	L	V	**r (1.8)**	S (1.1)	S (0.9)	[54, 118]
Region III (805 – 845)						
K805Q	K	Q	S (1)	**R (2.2)**	S (0.18)	[75, 119, 120]
A809V	A	V	**R (2.6)**	S (1.7)	**R (6.3)**	[58, 88, 108]
V812L	V	L	**R (2.5)**	**R (3.2)**	**R (2.9)**	[104, 118, 131]
T813S	T	S	**R (2.5)**	**R (2.7)**	**R (4.9)**	[108]
T821I	T	I	**R (4.5)**	**r (1.9)**	**R (21)**	[75, 119, 120]
P829S	P	I	**R (2.0)**	S (1.6)	S (1.1)	[54, 118]
A834P	A	P	**R (5.4)**	**R (3)**	**R (6.4)**	[41, 50, 57, 58, 103, 125]
T838A	T	A	S (1.8)	S (0.8)	**R (2.4)**	[104]
G841A	G	A	**R (3.2)**	**R (2.6)**	**R (4.3)**	[41, 98, 108]
G841S	G	S	**R (2.2)**	S (1.1)	**R (2.1)**	[130]
M844T	M	T	S (1.4)	S (1.3)	**R (2.5)**	[135]
M844V	M	V	**R (2.5)**	**r (1.6)**	**R (2.2)**	[135]
Outside conserved region						
L862F	L	F	**r (1.7)**	S (0.9)	S (1.1)	[54, 118]
V946L	V	L	S (1.1)	S (0.9)	**R (2.4)**	[54, 118]
L957F	L	F	**R (2.7)**	S (1.4)	S (1.3)	[54, 118]

Region V (978 – 988)						
del981-2	DL	Del 2	**R (8.3)**	**R (2.8)**	**R (3.6)**	[58, 71, 113, 122]
A987G	A	G	**R (5.3)**	**R (11.3)**	S (1.2)	[41, 50, 88, 102, 103, 119]

Boldface indicates the mutations conferring resistance; Deletions start at the designated codon and continue through the number of shown codons; R, resistant strain (≥2 fold reduction in susceptibility to antiviral drugs); r, low-grade resistance or <2 fold reduction in susceptibility to antiviral drugs; S, sensitive strain; NA, not available; NT, not tested.

a) The level of resistance of each mutation is expressed as the ratio of the half maximal inhibitory concentration (IC_{50}) of the mutant to that of drug – sensitive wild-type (IC_{50} of mutant/IC_{50} of wild type ratio);

CHAPTER V – New strategies for HCMV management

New treatment options for HCMV infection in immunocompromised patients are urgently needed because the currently available drugs have major limitations, illustrated by their toxicities (hematotoxicity for VGCV, nephrotoxicity for FOS and CDV) and the phenomenon of drug resistance. Given the paucity of current antivirals drugs, the emergence of HCMV resistance to antivirals constitutes a rising therapeutic challenge. Moreover, multidrug resistant HCMV infections remain a clinically challenging complication [136, 137].

In allo-HSCT recipients, the high level of cellular immunosuppression, including a deficit in number and function of HCMV-reactive T cells, deeply impairs the host response towards HCMV active infection and constitutes a risk factor for the emergence of HCMV drug resistance [138]. In this context, adoptive transfers of HCMV-specific T-cell could constitute a beneficial strategy, but still remains logistically complicated [139, 140].

Three novel oral drugs targeting HCMV replication are being evaluated in clinical trials. Brincidofovir (hexadecyloxypropyl-CDV; CMX001), the lipid conjugate of CDV, has been reported to be less nephrotoxic than CDV [141] and to prevent efficiently the incidence of HCMV events in HSCT recipients [142]. Maribavir (MBV) is a benzimidazole riboside that impairs viral encapsidation and nuclear egress of viral particles through the direct inhibition of HCMV UL97 phosphotransferase. Despite promising previous results, a low-dose phase III MBV study failed to prevent HCMV disease in allo-HSCT recipients [143]. However, few reports suggest some efficacy of high-dose MBV treatment as salvage therapy [144, 145]. MBV has completed a phase II trial for the treatment of resistant or refractory HCMV infections in transplant recipients, but results are still unknown (clinicaltrials.gov NCT01611974). Letermovir (AIC246, MK-8228) is a highly selective anti-HCMV agent that inhibits the viral terminase complex [146, 147]. Two successful phase II clinical studies demonstrated the efficacy of letermovir for HCMV prevention in transplant recipients [148, 149]. However, one letermovir resistance mutation has been evidenced in a case of HCMV breakthrough during prophylaxis [150]. Letermovir has recently entered a phase III study for the prevention of HCMV infection in adult allo-HSCT recipients (clinicaltrials.gov NCT02137772).

The use of drugs targeting cellular pathways, rather than HCMV replication, may constitute an alternative for the treatment of HCMV infections. The antimalarial agent artesunate has an anti-HCMV activity through the downregulation of NF-kB and Sp1 signaling pathways [151]. Artesunate has been used in salvage situations with mixed results [152]. Leflunomide, an immunosuppressive agent used for the treatment of rheumatoid arthritis, inhibits HCMV replication by preventing tegument acquisition by viral nucleocapsids [153]. This drug, alone or in combination, has potential utility in the treatment of complex HCMV syndromes [154]. Finally, the use of immunosuppressive agents mTOR inhibitors (i.e., sirolimus and everolimus) in transplant recipients has been associated with a lower incidence of HCMV infections [155]. These molecules seem to inhibit the later stages of HCMV replication [156]. Moreover, mTOR inhibitors may be beneficial in transplant recipients with GCV-resistant HCMV [157]. However, no systematic evaluation of these different drugs targeting cellular pathways has been performed for HCMV treatment.

The development of novel anti-HCMV molecules with improved efficacy and lower toxicity, targeting different viral proteins, together with the possibility of alternative options involving cellular metabolic pathways, will provide new possibilities of combination therapy. This strategy, that has proven to be highly effective for the treatment of HIV or HCV infections, is now required for the therapeutic management of HCMV infection in transplant recipients, since the incomplete viral suppression obtained with a single agent may not be supplied by the host immune system.

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