



The translational Research platform RegaVir

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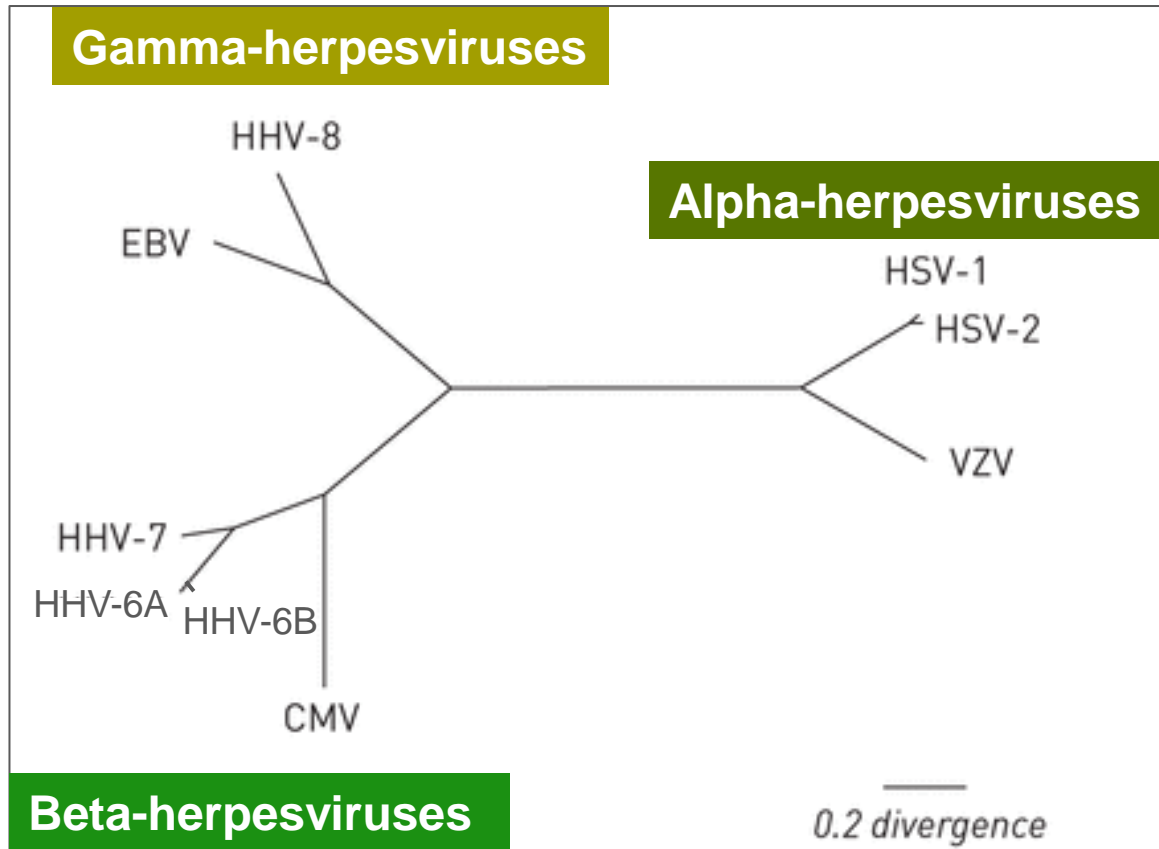
RegaVir: Research Group for Antiviral Resistance



AIMS

- Provide rapid **genotyping and/or phenotyping** of clinical isolates of **herpesviruses** recovered from **immunocompromised patients** who **fail antiviral therapy** to:
 - determine viral drug-resistance as reason for failure of therapy
 - optimize antiviral therapy
 - avoid drug toxicity
 - improve patient care
 - reduce costs of antiviral treatment
- Get insights into **herpesvirus diversity & evolution** in the immunocompromised host

Phylogenetic tree of human herpesviruses (HHVs)



- Order **Herpesvirales**
- Family **Herpesviridae**
 - **Alphaherpesvirinae**
 - **Betaherpesvirinae**
 - genus *Cytomegalovirus*
 - genus *Roseolovirus*
 - **Gammaherpesvirinae**
 - genus *Lymphocryptovirus*
 - genus *Rhadinovirus*

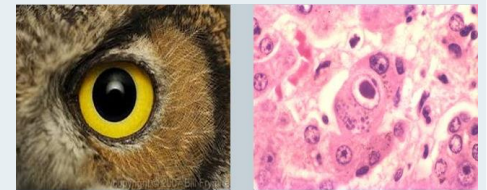
All human herpesviruses (HHVs) can lead to severe disease among immunocompromised (IC) patients, due to primary infection, reactivation or re-infection

Human Herpesviruses (HHVs)

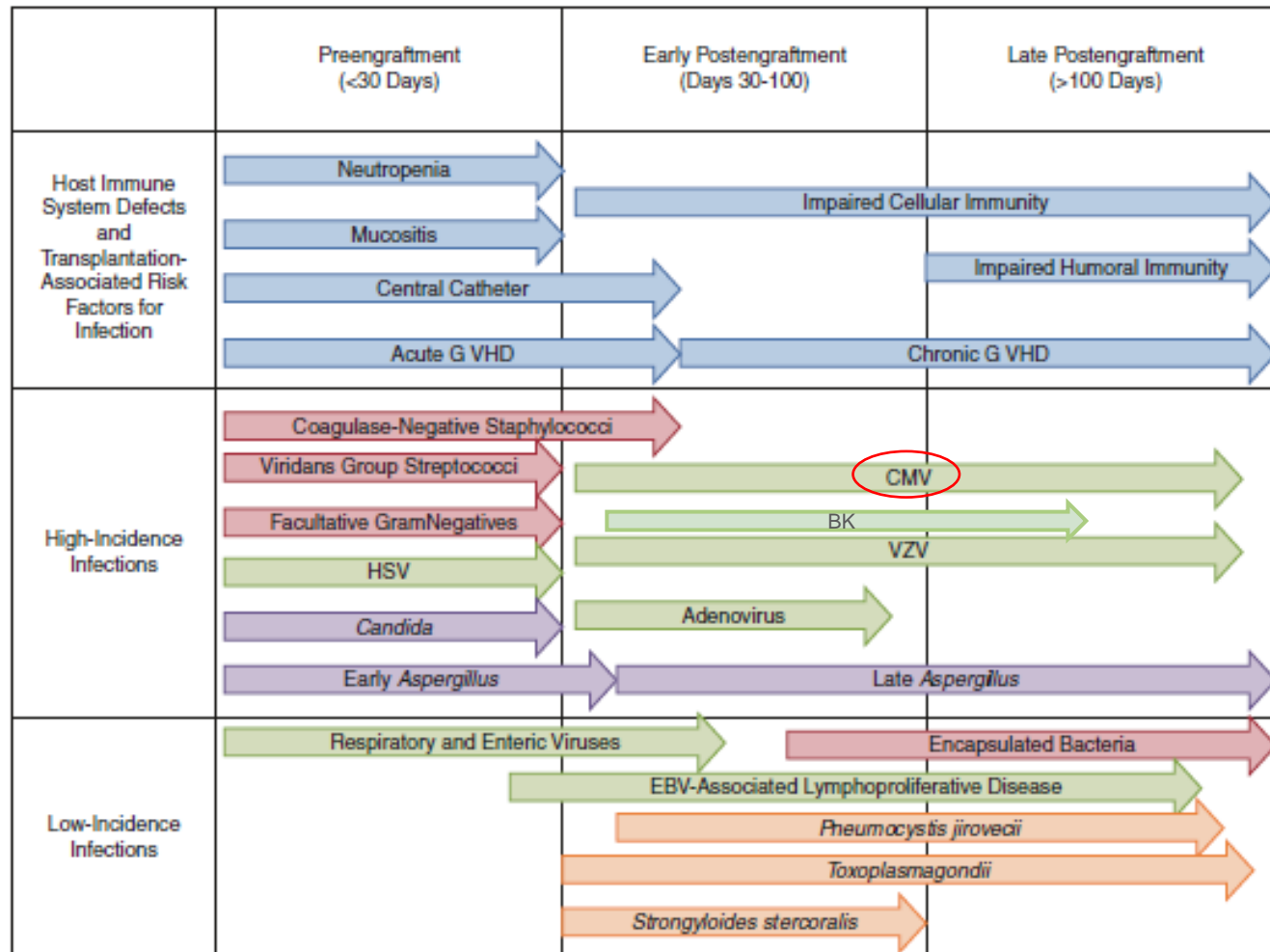
Designation	Common name	Subfamily	Genome size (kb)
HHV-1	Herpes simplex type 1 (HSV-1)	α	152
HHV-2	Herpes simplex type 2 (HSV-2)	α	155
HHV-3	Varicella-zoster virus (VZV)	α	125
HHV-4	Epstein-Barr virus (EBV)	γ	172-173
HHV-5	Cytomegalovirus (CMV)	β	227-236
HHV-6A HHV-6B	Human herpesvirus 6A Human herpesvirus 6B	β	159-162
HHV-7	Human herpesvirus 7	β	144-153
HHV-8	Human herpesvirus 8 Kaposi's sarcoma associated virus	γ	134-138

Human Herpesviruses (HHVs)

Subfamily	Biological properties
<p>α-Herpesvirinae HSV-1, HSV-2, VZV</p>	<p>Fast growth and spread in cell cultures Short replication cycle Lytic infection in fibroblasts and epithelial cells Latent in neurons</p>
<p>β-Herpesvirinae HCMV HHV-6A, HHV6B, HHV-7</p>	<p>Slow grow in cell culture Long replication cycle</p> <p>Cytomegalovirus</p> <ul style="list-style-type: none"> - Grows in many \neq cell types - Enlarged cell large with large cytomegalic inclusion “owl eyes” within the nuclei of infected cells - Latent in myeloid lineage hematopoietic cells - Shed from kidney and salivary gland <p>Roseolovirus</p> <ul style="list-style-type: none"> - Grows in T lymphocytes, salivary gland - Latent in macrophages, lymphocytes
<p>γ-Herpesvirinae EBV, HHV-8</p>	<p>Grows in epithelial cells Latent in B cells Lymphoproliferative Associated with malignancies</p>



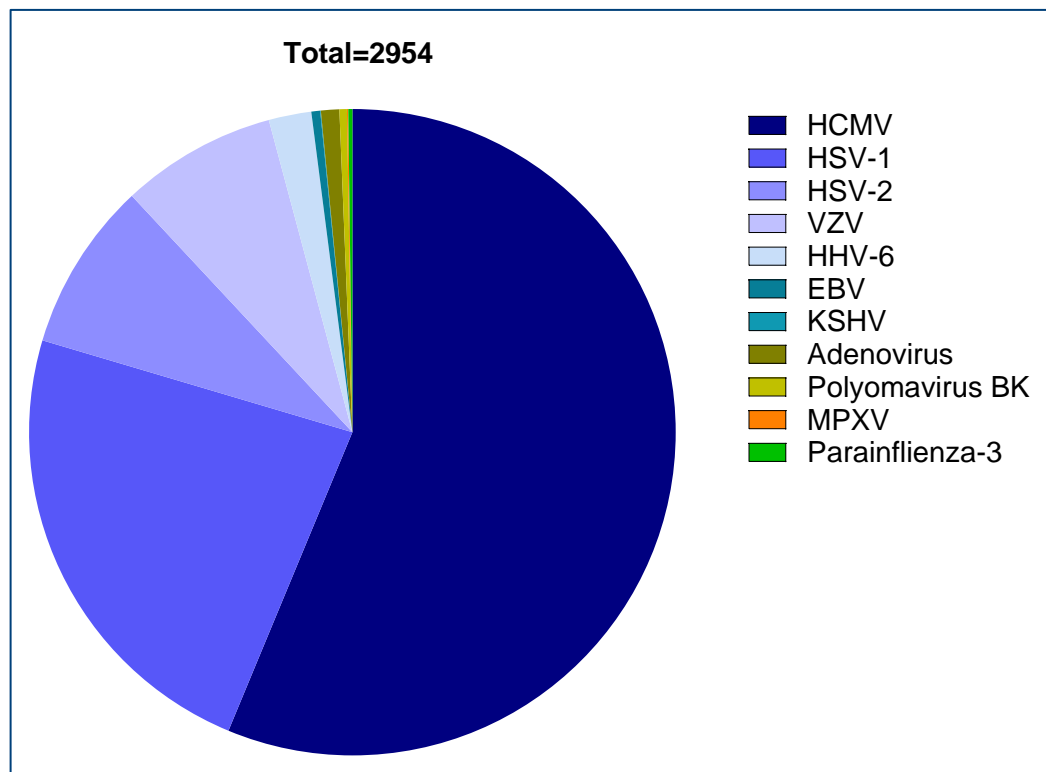
Timeline of common post-transplant infections



Pereira et al, 2019

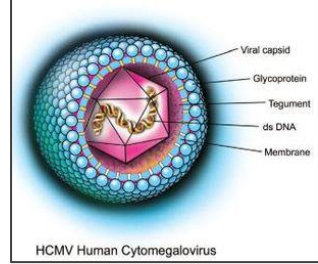
CMV is one of the clinically most significant viral pathogens causing infections in immunocompromised patients, especially in HSCT recipients.

Tests requested to RegaVir

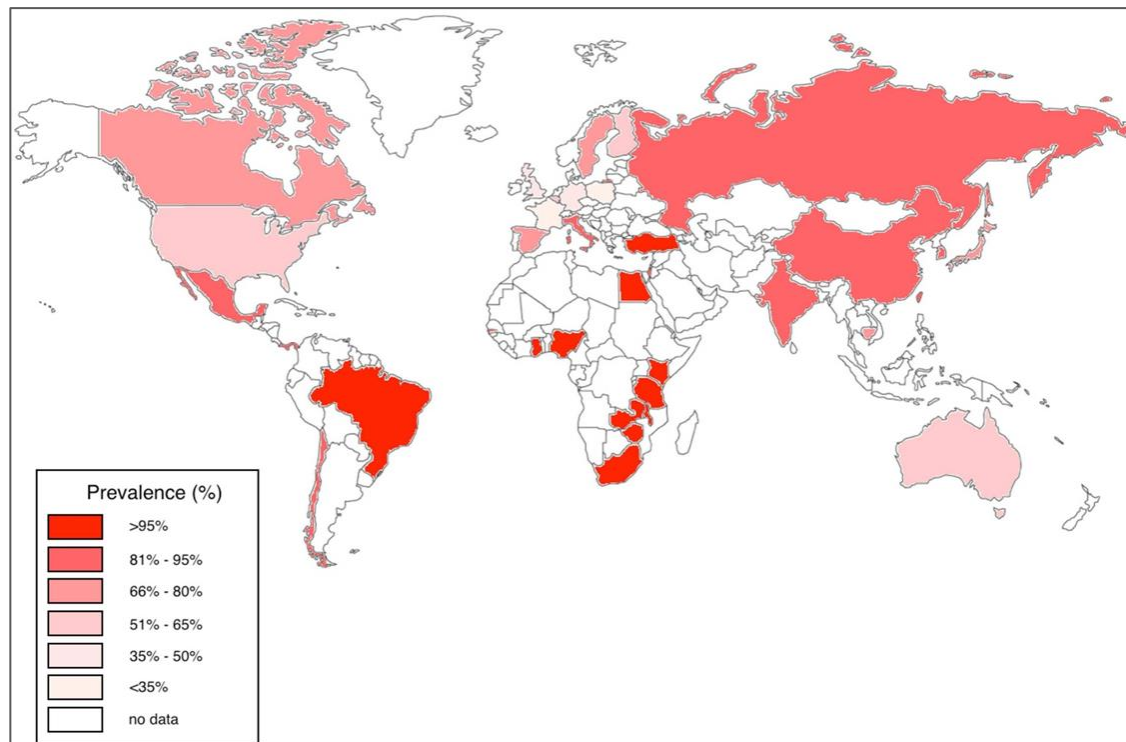


Virus	N° of tests requested	Percentage
HCMV	1662	56.3%
HSV-1	689	23.3%
HSV-2	250	8.5%
VZV	230	7.8%
HHV-6	63	2.1%
EBV	13	0.44%
KSHV	1	0.03%
Adenovirus	27	0.91%
Polyomavirus BK	11	0.37%
MPXV	2	0.07%
Parainfluenza-3	6	0.20%

Human cytomegalovirus (HCMV)



- **A wide-spread virus** infecting between 60% to 70% of adults in industrialized countries and close to 100% in emerging countries.
- **HCMV seropositivity >> among adults with risk factors for acquisition of HIV infection** (e.g., MSM) than in the general population.



- ✓ *In the USA, Australia and Europe, CMV seroprevalence among adults is variable, estimated (between 36 and 77%).*
- ✓ *CMV is highly endemic in developing countries, particularly in sub-Saharan Africa, with a seropositivity rate often approaching 100% in adults.*

HCMV infection is complex with 3 ≠ subtypes of infection

- Primary infection
- Reinfection (superinfection)
- Reactivation

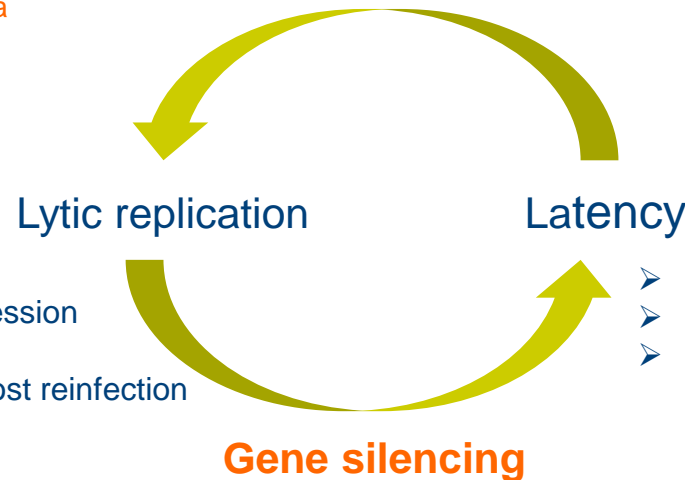
All associated with significant morbidity and mortality

α: physical or emotional stress, local tissue injury, UV radiation, immune suppression

β: immune suppression, inflammation

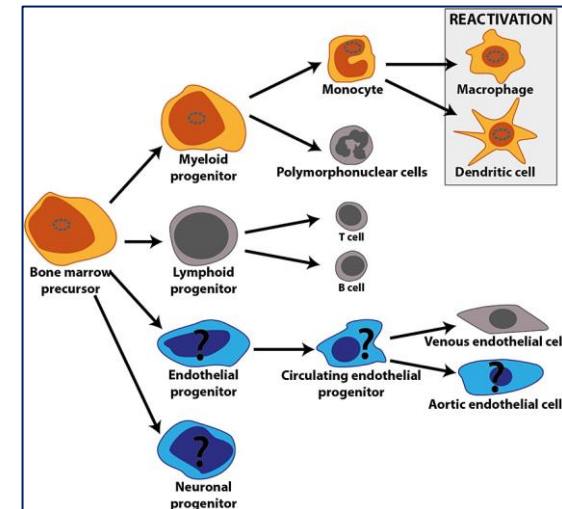
γ: physical or emotional stress, immune suppression, differentiation to plasma cells, hypoxia

Reactivation

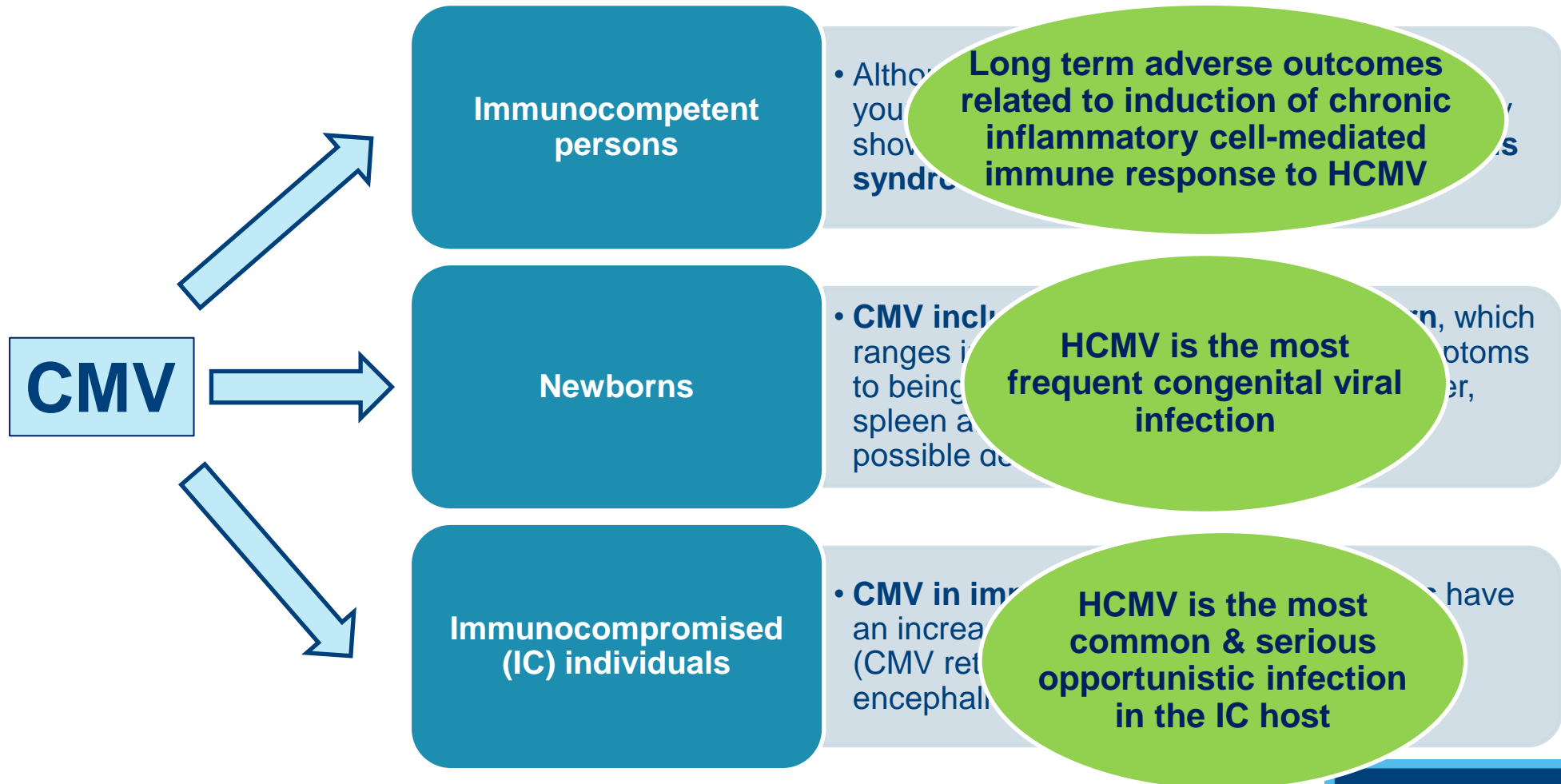


- Temporal regulation gene expression
- Production infectious virus
- Dissemination to new hosts / host reinfection

- No or limited gene expression
- No production progeny virus
- Lifelong presence in the host



Outcome of HCMV infection depends very heavily on the immune status of the patient



Human Cytomegalovirus (HCMV)

- **A significant pathogen among:**

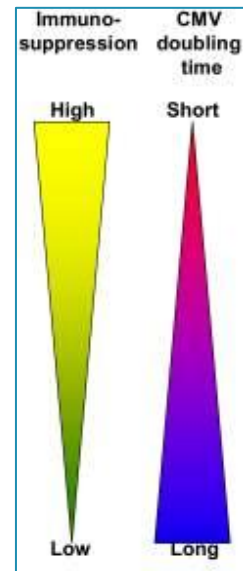
Solid organ transplant (SOT) recipients	Allogeneic hematopoietic stem cell transplant (HSCT) recipients
HIV infected individuals	Patients under Chemotherapy and/or radiotherapy
Patients receiving immunomodulatory drugs	Patients with inborn errors of immunity (IEI)

cCMV: congenital cytomegalovirus

Neonates congenitally infected with CMV (**cCMV**)

Infection *in utero*: Leading cause of infectious disease related birth defects

- **1 in 100 infected; 1 in 1000 present symptoms/pathology**
- Mild to severe hearing loss
- Cognitive deficits
- Physical abnormalities



Boeckh & Ljungman, 2009

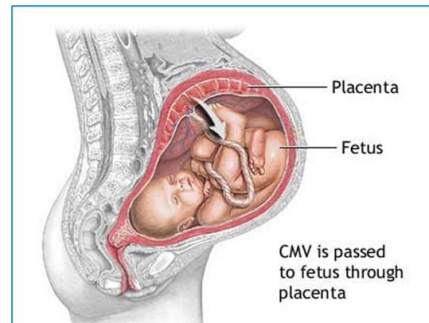
HCMV transmission

Via close, intimate contact with a person who is excreting virus in:

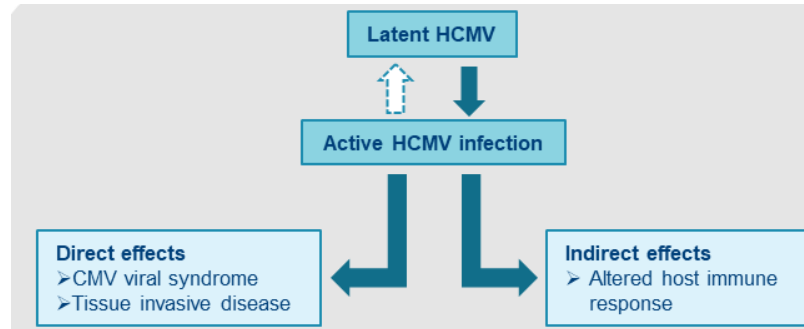
- Saliva
- Urine
- Other bodily fluids: semen, cervical secretions, breast milk, tears)

It can be transmitted:

- Sexually
- Orally
- Via respiratory droplets
- Food and drink sharing
- **Transplanted organs**
- **Blood transfusions**
- **In utero (transplacental)**
- At birth (intra partum)
- Through breast feeding



Impact of HCMV on transplant outcomes

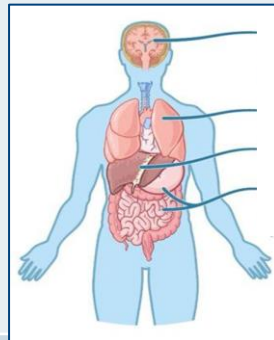


Direct effects

CMV syndrome

Tissue-invasive CMV disease

- Gastrointestinal disease
- Pneumonitis
- Hepatitis
- CNS disease
- Retinitis
- Nephritis
- Pancreatitis
- Myocarditis



Mortality

Indirect effects

Acute allograft rejection

Chronic allograft rejection

Opportunistic and other infections

- Fungal superinfection
- Bacterial superinfection
- EBV and PTLD
- Hepatitis C recurrence
- Other viruses (HHV-6, HHV-7)

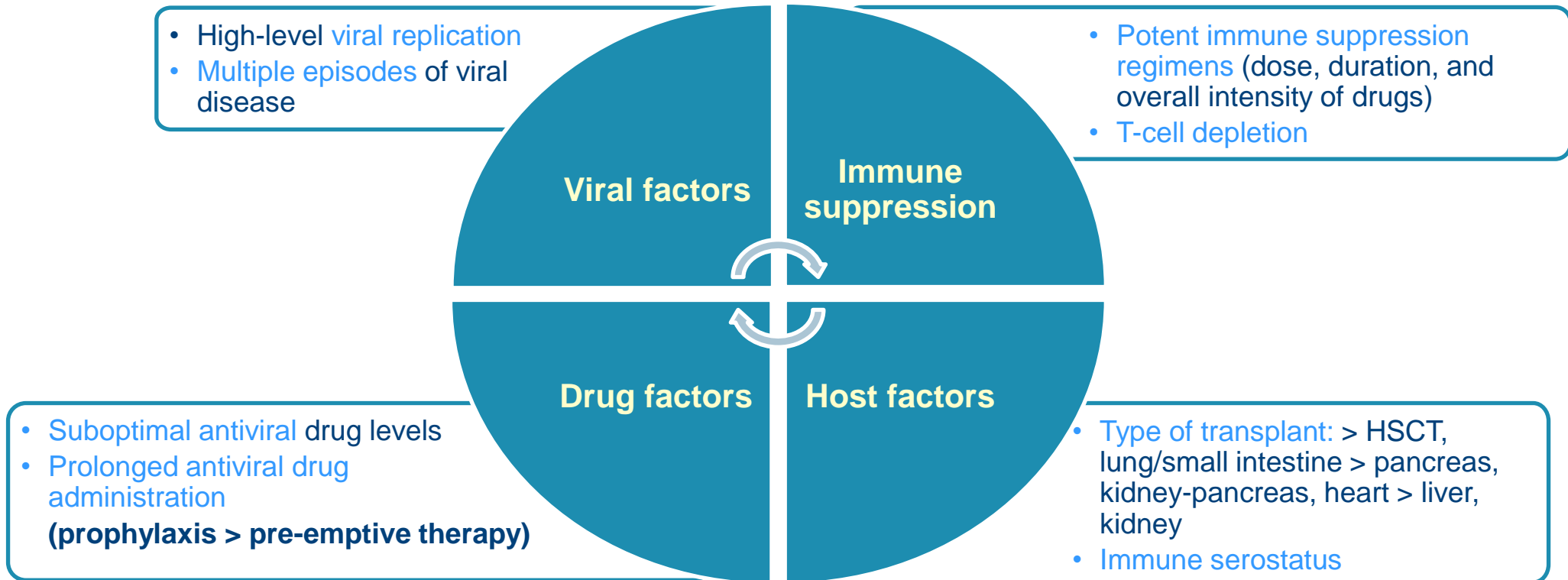
New onset diabetes mellitus

Malignancies

Thrombosis

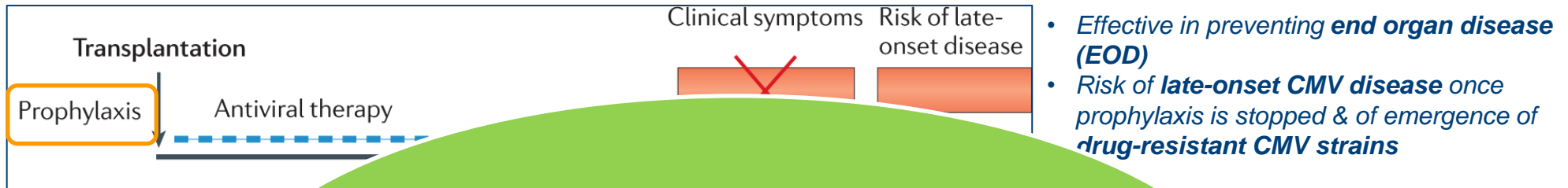
Mortality

Risk factors for HCMV drug-resistance



Risk category	Type	Donor/Recipient Immune status
High	Primary infection	D+ /R-
Intermediate	Reactivation	D- /R+
Intermediate	Superinfection	D+ /R+
Low	Risk with exposure	D- /R-

Two distinct strategies used to reduce human cytomegalovirus disease in allograft recipients



No universal agreement among transplant centers regarding:
Prophylaxis versus pre-emptive therapy

- **Prophylaxis:** an anti-CMV drug (as soon as the patient can tolerate oral medication) for a period of 3-6 months (or 3-6 months for SOT recipients).

Pre-emptive therapy (PET)

Advantages

- Minimizes drug exposure
- Potentially decrease toxicity and cost
- Theoretically, lower risk of resistance
- Less late-onset disease: may allow development of cell-mediated immune response

Disadvantages

- More difficult to coordinate
- May not eliminate the indirect effects of CMV
- May be unsuccessful in preventing progression to active disease in high-risk patients

Antiviral prophylaxis

Advantages

- Very effective at preventing CMV infection & disease
- Better evidence to diminish CMV indirect effects
- Logistically more feasible, but still requires frequent monitoring of adverse events

Disadvantages

- Higher drug costs; lower laboratory monitoring tests
- Drug toxicity: more frequent adverse events
- Development of drug-resistance
- Higher risk of late-onset CMV disease (D+/R- are highest risk patients)

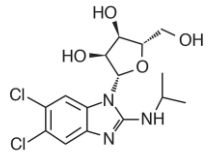
Late onset CMV disease

- CMV disease occurring > 3 months post-transplant.
- May be primary infection (D+/R-) or recurrence (R+).
- In epidemiological studies, associated with significant morbidity (including graft dysfunction) and occasional mortality (indirect effects).
- Incidence 3%-17%.
- **Prophylaxis: how do we deal with late onset disease?**
 - ✓ Prolong prophylaxis?
 - ✓ Use better prophylaxis?
 - ✓ Perform careful virologic monitoring of high-risk patients after completing prophylaxis?
 - ✓ Monitor cellular host-immune response during or after prophylaxis?

Licensed anti-herpesvirus drugs

	DNA polymerase inhibitors					Terminase inhibitor	UL97 PK inhibitor
	Acyclovir Valacyclovir	Penciclovir Famciclovir	Ganciclovir Valganciclovir	Cidofovir	Foscarnet	Letermovir	Maribavir
HSV-1 (HHV-1)	1 st line	approved		resistance	resistance		
HSV-2 (HHV-2)	1 st line	approved		resistance	resistance		
VZV (HHV-3)	1 st line	approved		resistance	resistance		
EBV (HHV-4)			off-label	off-label	off-label		
HCMV (HHV-5)			1 st line	approved	approved	approved for prophylaxis	orphan Drug Designation
HHV-6A			off-label	off-label	off-label		
HHV-6B			off-label	off-label	off-label		
HHV-7			off-label	off-label	off-label		
KSHV (HHV-8)			off-label	off-label	off-label		

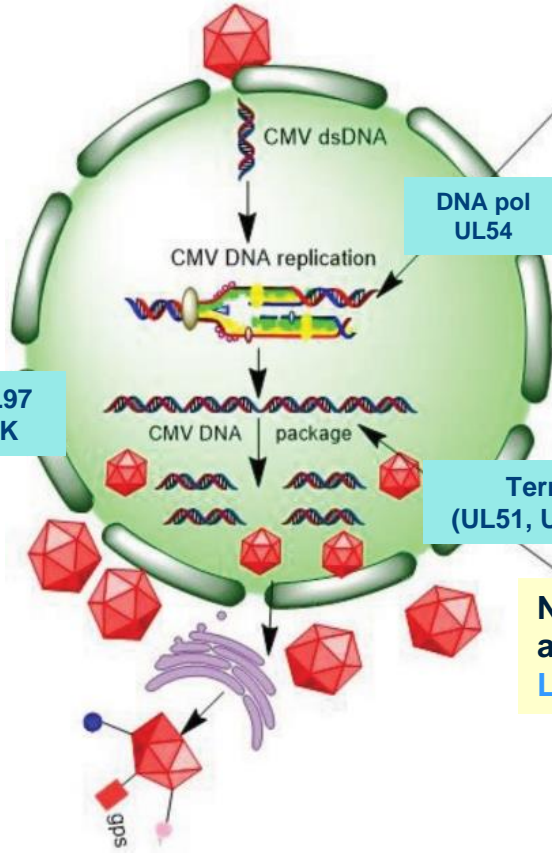
Anti-HCMV drugs



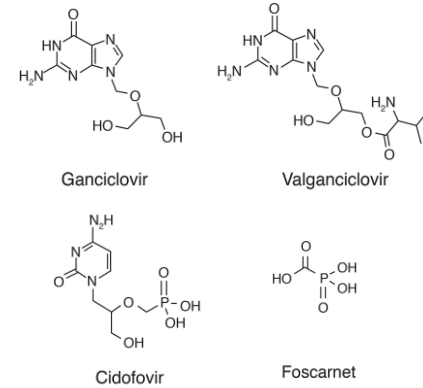
Benzimidazole riboside
Maribavir

DNA elongation
DNA packaging
Capsid egress

UL97
PK



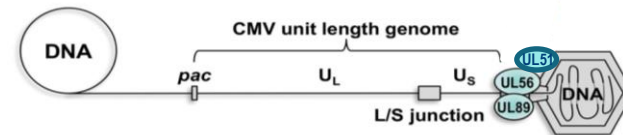
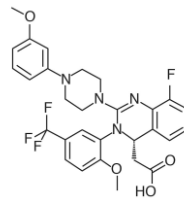
Nucleoside analogues
Ganciclovir (GCV) / valganciclovir (VGCV)
Nucleotide analogues
Cidofovir (CDV)
Pyrophosphate analogues
Foscarnet (PFA)



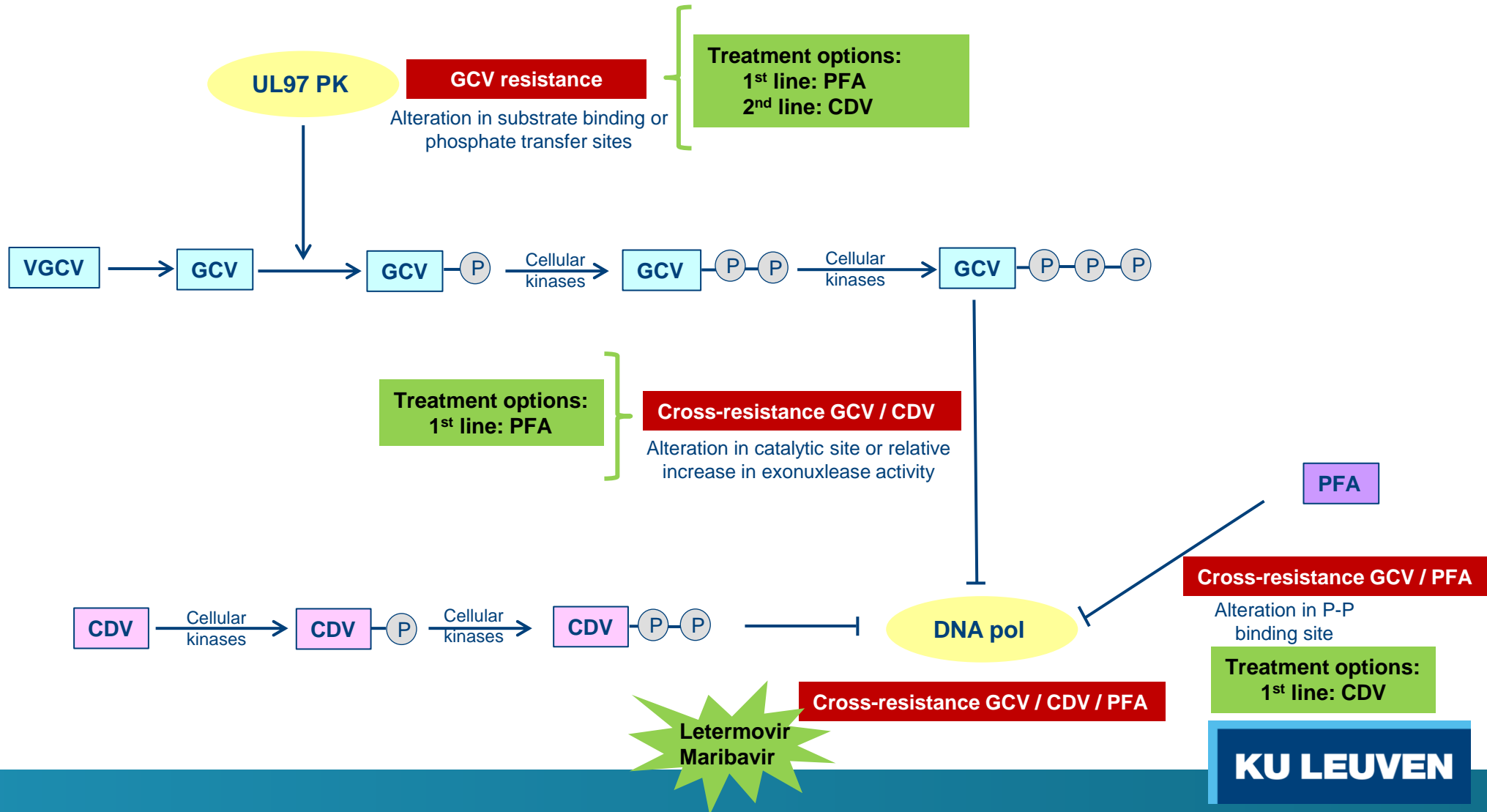
Viral genome replication

Cleavage of viral DNA into unit length genomes

Non-nucleoside analogue
Letermovir



Mechanisms of drug-resistance in HCMV



Why treatment with antiviral agents may lead to clinical failure?

- Poor drug-compliance
- Pharmacological factors:
 - poor drug absorption
 - incorrect dosage
- **Viral drug-resistance**

➔ Clinical drug-resistance \neq virological drug-resistance

Fundamental to
evaluate
virological drug-
resistance

- **Risks factors for emergence of drug-R:**
 - prolonged (several months) antiviral therapy with viral replication in the presence of the drug
 - suboptimal drug levels
 - high levels of immunosuppression
 - lack of CMV immunity

Recommendations for drug-R HCMV testing

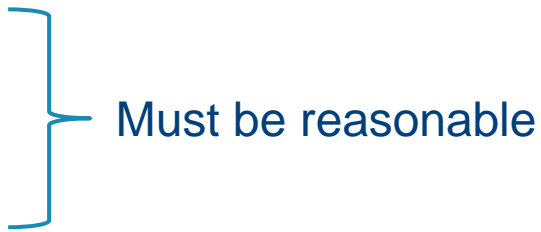
- ***SOT recipients***

- When unchanged or increasing HCMV viral loads or unresolved CMV disease is seen after at least 2 weeks of antiviral therapy at an appropriate dose or > 6 weeks of GCV exposure.

- ***HSCT recipients***

- When the viral load declines < 10-fold after > 2 weeks of antiviral therapy at an appropriate.

Diagnostic techniques for HCMV drug-resistance

- Genotypic resistance testing is indicated when there is a **rising viral load while on therapy of extended duration.**
- Reliable testing requires:
 - Clinical sample with **sufficient HCMV DNA content**
 - **Efficient pre-processing** (DNA extraction, enrichment and/or amplicification)
 - **Appropriate sequencing** of the regions where the diagnostic mutations develop.
- To be clinically useful:
 - Turn-around time
 - Logistical complexity
 - Cost

Must be reasonable

Prevalence and consequences of HCMV drug-resistance

- Associated with **progressive disease and treatment failure**: cause significant morbidity and mortality
 - **Immunocompromised patients**: 1% to 13% among SOT recipients (> lung, small bowel transplants).

- Although the prevalence of resistance is low, the **impact of drug resistant CMV infections on patient outcomes is high** ⇒ **genotypic testing** is recommended when resistance is suspected.

- HCMV-drug-R is associated with:
 - Higher rates of hospitalization
 - Increased length of hospital stay
 - Higher costs
 - Increased adverse events from alternative therapies (especially foscarnet & cidofovir)
 - Increased rates of rejection and allograft loss
 - Increased mortality

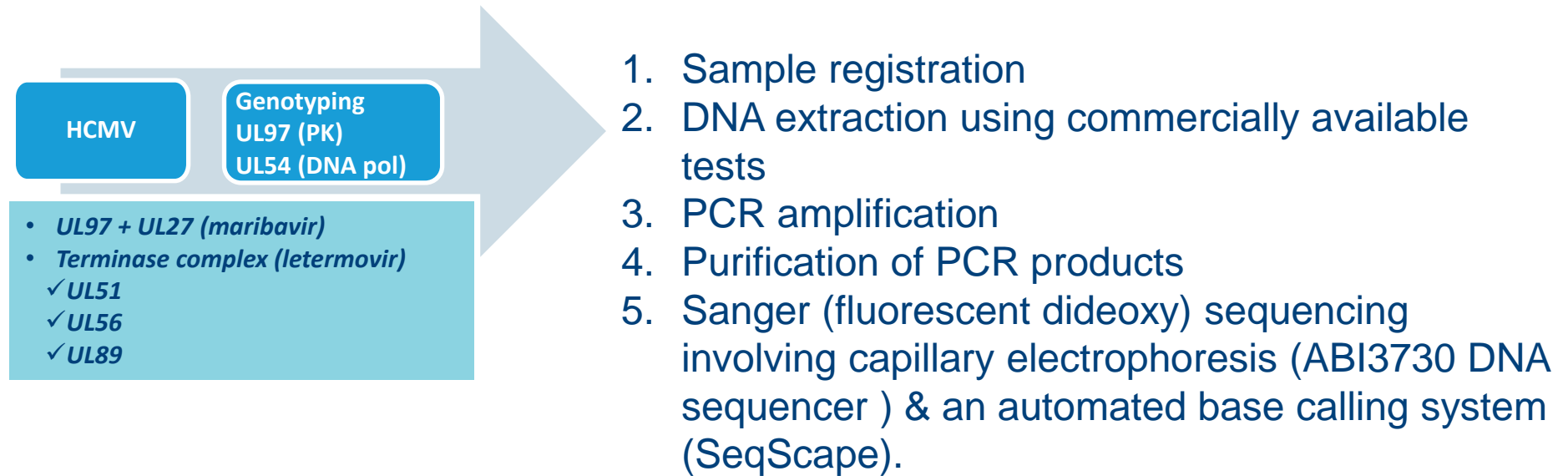
Prevalence of HCMV drug-resistance mutations

- Study by Steven B Kleiboeker (2023) analyzing 2750 patient samples:
 - 826 samples **(30.04%)** had resistance to one or more anti-CMV drug.
 - Resistance mutations were most common in **UL97** (27.64% GCV-R & 9.96% MBV-R).
 - Resistance mutations in **UL54** were less common, with 6.11%, 5.98% and 1.76% of samples having GCV, CDV and FOS mutations, respectively.
 - For **LMV**, resistance mutations in UL56 were present in **7.17% of samples**, with mutations at **codon 325** representing 80.95% of the observed LMV resistance mutations.
 - Resistance to two drugs in 215 samples
 - Resistance to 3 or more drugs in 35 samples.
 - High prevalence of **CMV resistance mutations** - samples submitted from patients with suspected resistant CMV strains.
- For patients with suspected resistant CMV strains, **rapid monitoring for resistance allows treatment modifications based on objective results rather than empiric drug selection** - particularly relevant given the presence of mutations conferring resistance to more than one drug.

Herpesviruses drug-resistance

- Virologists have to provide clinicians with **fast and reproducible drug-resistance** diagnosis.
- There is a **restricted number of active antivirals** against herpesviruses and a limited number of viral targets.
 - **limited options for alternative treatments** in case of emergence of resistant viruses in IC patients.
 - most anti-HCMV are associated with important **side-effects**.
- In case of clinical evidence of resistance to the current available treatments, **monitoring of emergence of resistant viruses** is mandatory to **adjust antiviral therapy**.

HCMV antiviral resistance tests available at RegaVir

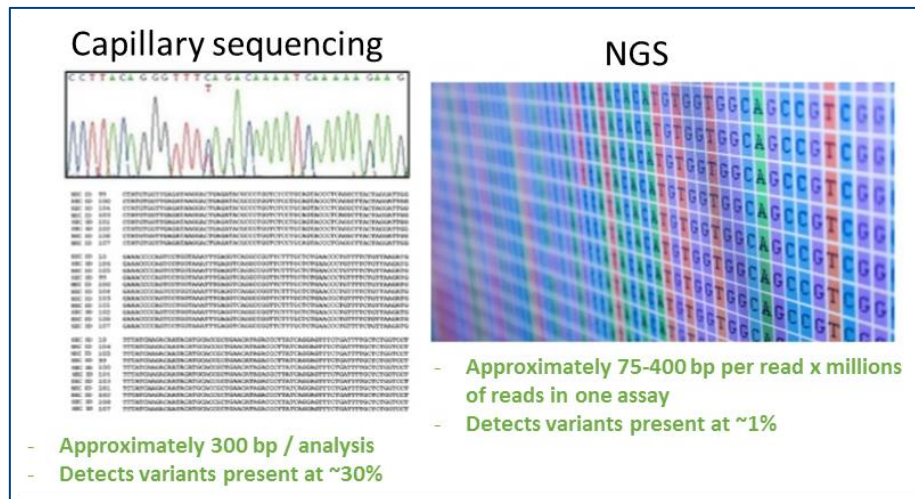


- We kindly ask to fill in a **“Test request form”**, available at www.regavir.org

HCMV antiviral resistance tests available at RegaVir

- **Herpesvirus genotyping**

- **Prospectively:** capillary (Sanger sequencing)
- **Retrospectively:** next-generation sequencing (NGS)



Limitations of capillary sequencing (Sanger) to detect viral minor populations → **targeted sequencing of viral genes with Illumina MiSeq (NGS)**

Reporting HCMV antiviral genotyping

- We provide **Reports (Intermediate/Final)** with:
 - Results on **genotyping** are provided as gene mutations associated with
 - natural genetic polymorphisms
 - drug-R mutations
 - novel mutations of unknown significance
 - Interpretation of results
 - Conclusions
 - Optional therapies - **guidelines for treatment**

Sample requirements

- Commonly done on whole blood or plasma samples, representing disseminated viral genomes.
 - Samples with a viral load ≥ 1000 -200 IU/ml are advised.
 - International units are traceable to CMV DNA WHO International Standard.
 - Conversion factor
 $1 \text{ IU/mL} = 1.72 \text{ copies/mL}$ (range of the assay is 150- 6,000,000 IU/ml or 2.18-6.78 log₁₀ IU/m).
 - Limit of Detection (LOD) is 150 IU/ml.
 - **Accuracy of detection of mutants decreases with lower viral loads.**

Sample requirements

- CMV pathogenesis includes **localized viral replication outside the systemic circulation** ⇒ evolution of resistance mutations may differ at specific tissue sites of HCCMV disease (**compartmentalization**).
 - Analysis of a tissue biopsy or localized fluid (ocular, CSF, etc) is recommended if there is progressive HCMV disease while on therapy, and no mutations are detected in plasma or blood.
 - **Longitudinal evaluation**

Type of samples

Type	Amount and preservation for shipment
Whole Blood / plasma / serum	3 to 5 ml collected in EDTA tube, do not freeze
Bone Marrow	1 ml minimum, collected in EDTA tube, do not freeze
Bronchial Lavage / Bronchial Wash	1 to 3 ml, collected in sterile screw-cap tube
CSF	1 ml minimum, submitted in sterile screw-cap tube
Pleural Fluid	1 ml submitted in sterile screw-cap tube
Tissue biopsy	place fresh biopsy in a sterile screw-cap tube, add a small amount of saline to keep moist
Upper Respiratory Aspirate NP aspirate, nasal aspirate, tracheal aspirate, etc.	instill 1 to 2 ml sterile saline into desired location and gently aspirate contents, place collected fluid into sterile screw-cap tube
Urine	5 to 10 ml sample collected in a sterile urinalysis container transfer to a 15 ml sterile screw-cap tube
Vitreous Fluid	place collected vitreous fluid into small sterile screw-cap tube
Viral Culture	culture supernatant or infected cells

Instructions for shipping samples

- Optimally, **pretreatment or early treatment samples** will enhance the diagnosis → provision of such samples is recommended
- All specimens must be labeled with the **patient's name and collection date**.
- A RegaVir **Test Request Form** must accompany each specimen.
- Please use a **separate Test Request Form** for each specimen when sending **multiple specimens**.
- The name and address of the **Requesting Doctor(s) / Laboratory** must be provided on the package. shipping samples

Instructions for shipping samples

- **IMPORTANT**
- After sample collection, please store the specimens a.s.a.p. cooled (max. 4°C) and **always ship refrigerated.**
- Longer storage on room temperature diminishes successful genotyping analysis.
- **Please, send recent and fresh samples.**

RegaVir platform for translational research

Compartmentalization:

- ≠ body sites at ≠ time points

Combination therapy

Dynamics & evolution:

- ≠ time points (longitudinal)

Heterogeneity:

- minor populations by NGS
- isolation of viral clones – competitive viral fitness

HHVs simultaneous or consecutive infections

HHVs

Infection of immune-privileged sites:

- Eye
- CNS

Multidrug-resistance to standard antiviral agents

Resistance to novel agents:

- Maribavir
- Letermovir

Novel mutations – phenotyping:

- viral isolate
- recombinant virus