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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

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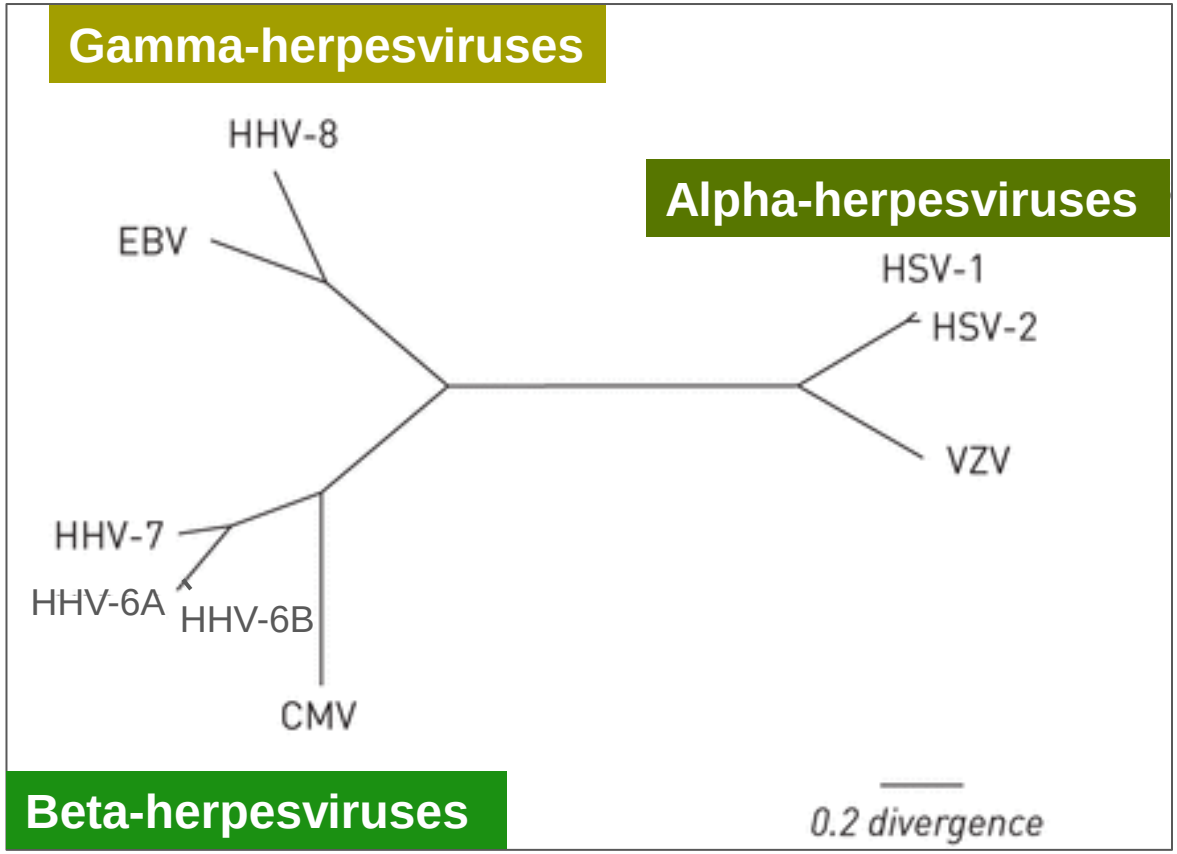
Phylogenetic tree of human herpesviruses (HHVs)



- 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

Adapted from Bori et al, 2012

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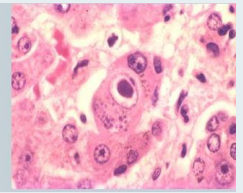
Human Herpesviruses (HHVs)

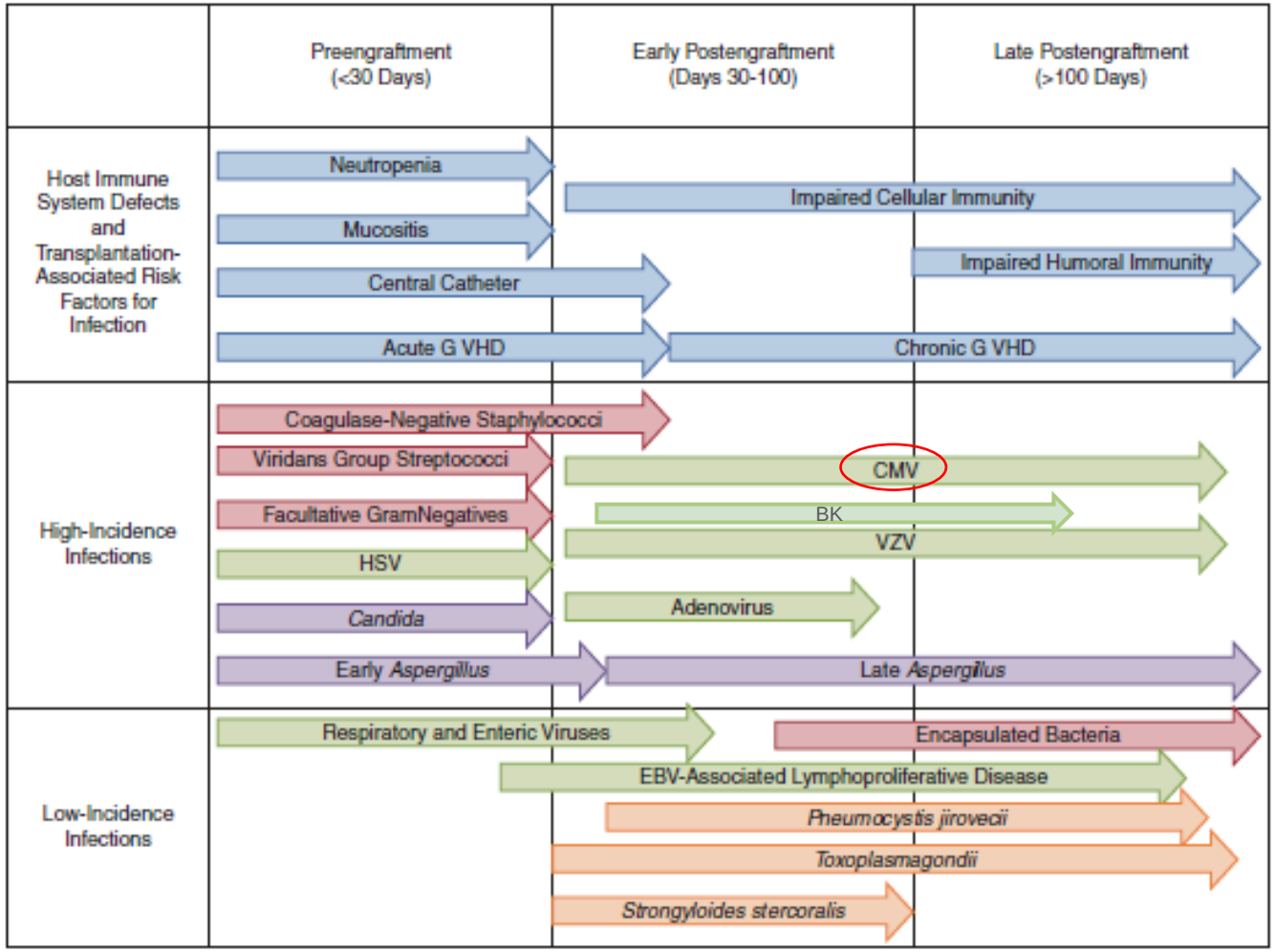
Designation	Common name	Subfamily	Genome size (kb)
HHV-1	Herpes simplex type 1 (HSV-1)	α	152
HHV-2	Herpes simplex type 2 (HSV-2)	α	155
HHV-3	Varicella-zoster virus (VZV)	α	125
HHV-4	Epstein-Barr virus (EBV)	γ	172-173
HHV-5	Cytomegalovirus (CMV)	β	227-236
HHV-6A HHV-6B	Human herpesvirus 6A Human herpesvirus 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
α-Herpesvirinae HSV-1, HSV-2, VZV	Fast growth and spread in cell cultures Short replication cycle Lytic infection in fibroblasts and epithelial cells Latent in neurons
β-Herpesvirinae HCMV HHV-6A, HHV6B, HHV-7	Slow grow in cell culture Long replication cycle **Cytomegalovirus** - Grows in many ≠ 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 **Roseolovirus** - Grows in T lymphocytes, salivary gland - Latent in macrophages, lymphocytes
γ-Herpesvirinae EBV, HHV-8	Grows in epithelial cells Latent in B cells Lymphoproliferative Associated with malignancies



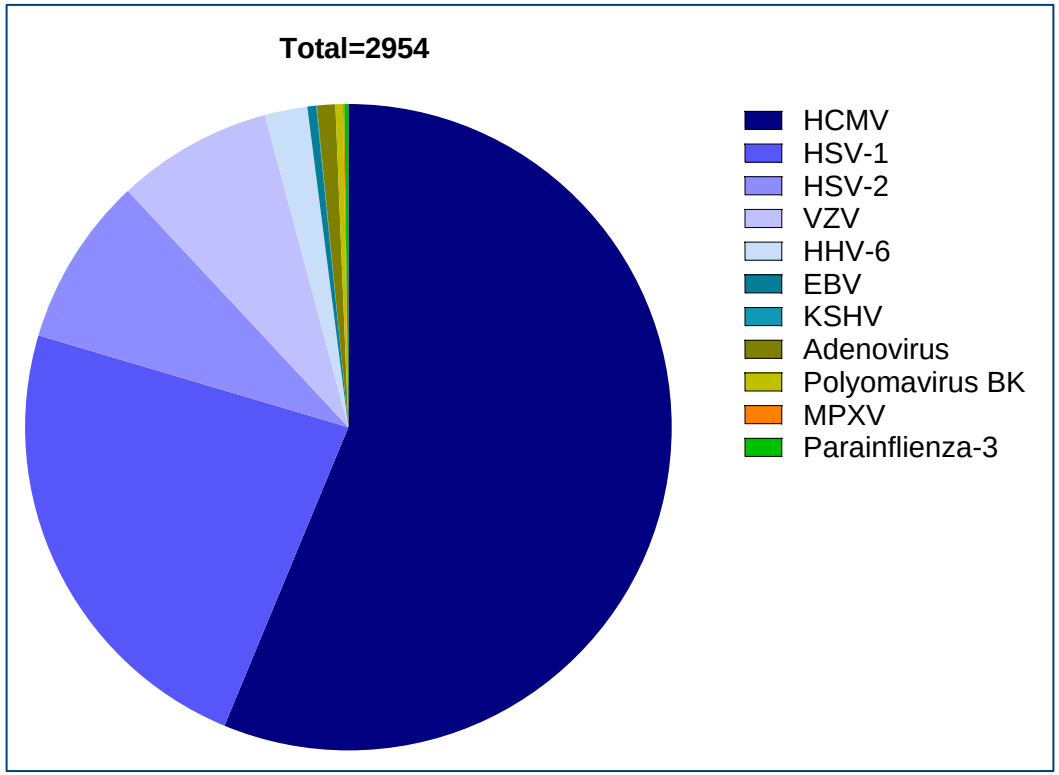
Timeline of common post-transplant infections



Pereira et al, 2019

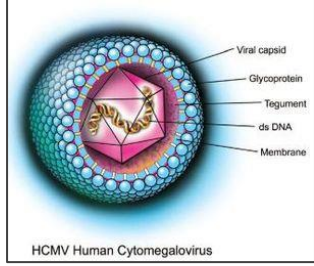
CMV is one of the clinically most significant viral pathogens causing infections in immunocompromised patients, especially in HSCT recipients.

Tests requested to RegaVir

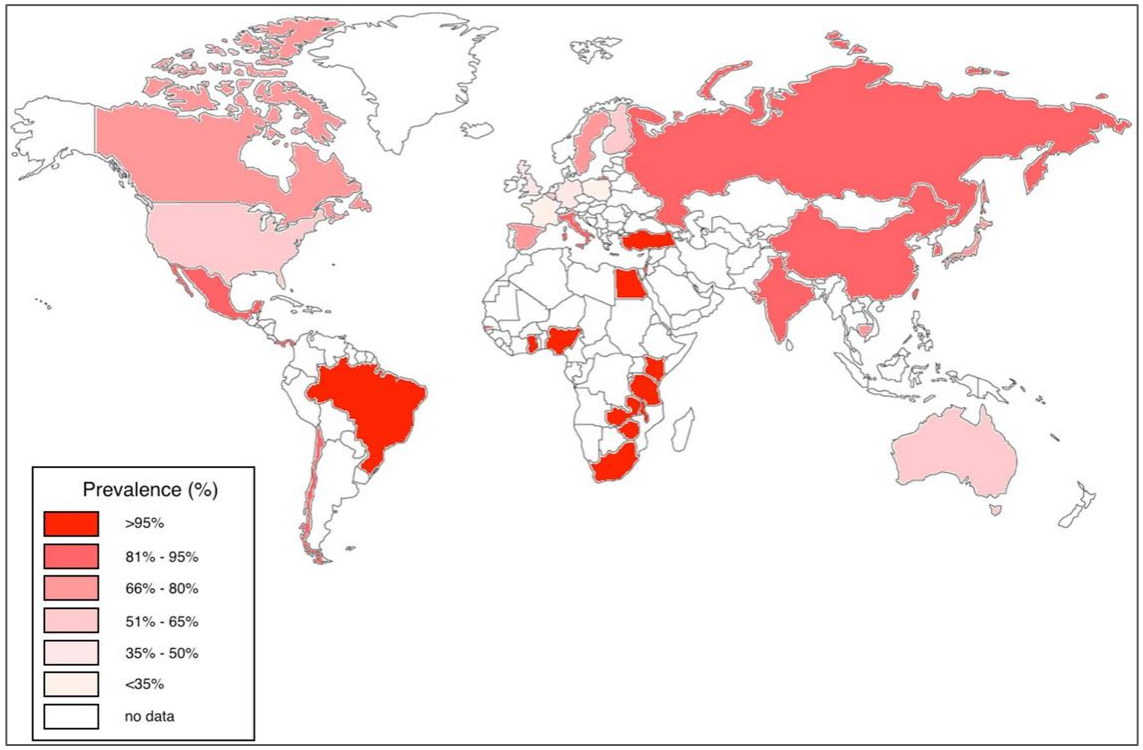


Virus	N° of tests requested	Percentage
HCMV	**1662**	**56.3%**
HSV-1	689	23.3%
HSV-2	250	8.5%
VZV	230	7.8%
HHV-6	63	2.1%
EBV	13	0.44%
KSHV	1	0.03%
Adenovirus	27	0.91%
Polyomavirus BK	11	0.37%
MPXV	2	0.07%
Parainfluenza-3	6	0.20%

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.*

Adland et al., 2015

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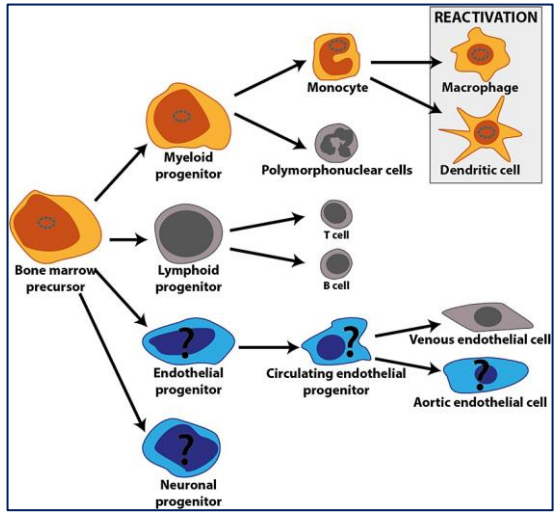
HCMV infection is complex with 3 ≠ subtypes of infection

- 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 suppresssion, differentiation to plasma cells, hypoxia

Reactivation

Lytic replication ⇄ Latency

Gene silencing

- 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

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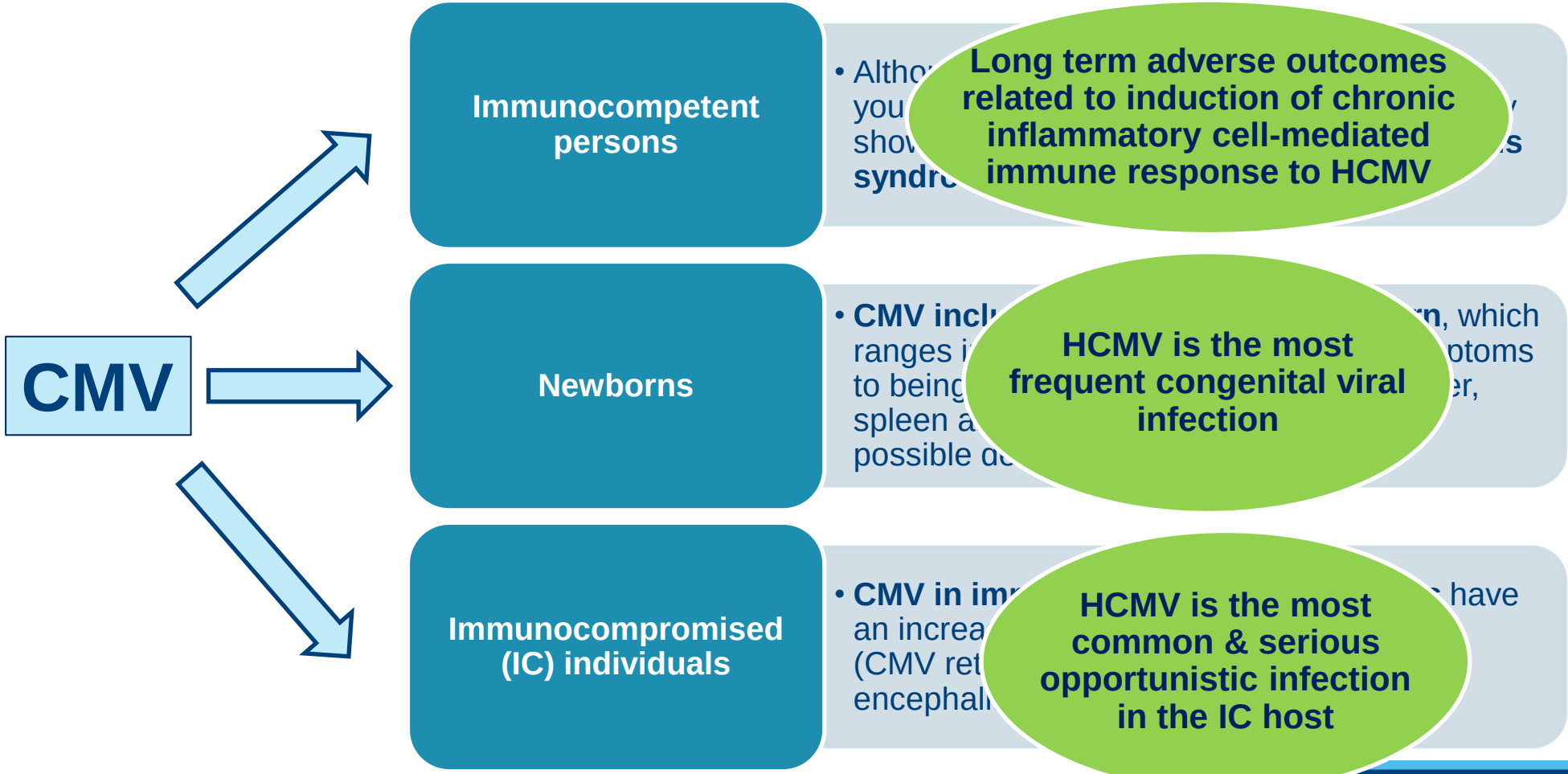
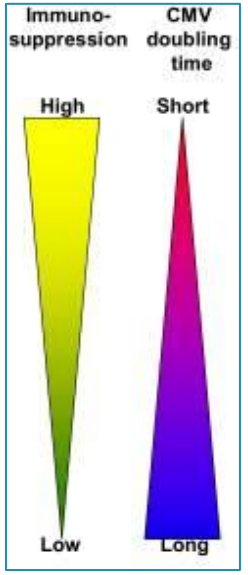
Outcome of HCMV infection depends very heavily on the immune status of the patient



Human Cytomegalovirus (HCMV)



Boeckh & Ljungman, 2009

- **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)**

- **IEI (inborn errors of immunity):** formerly called "primary immunodeficiencies" [PIDs])

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

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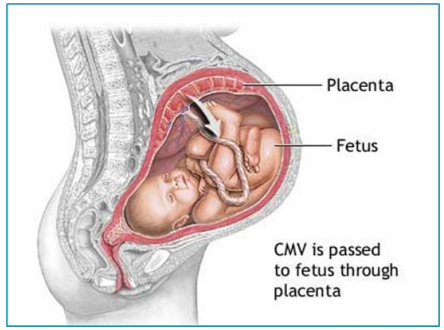
HCMV transmission

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



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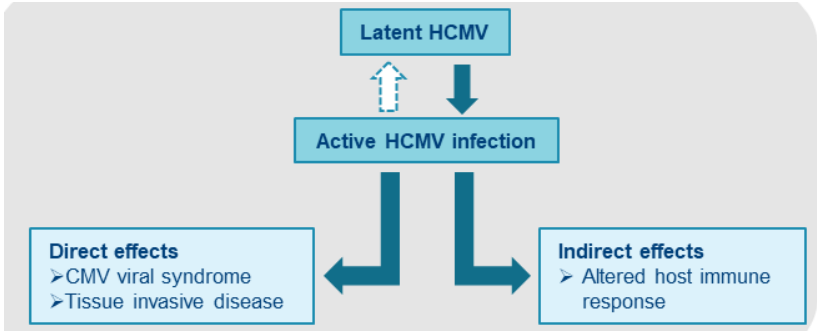
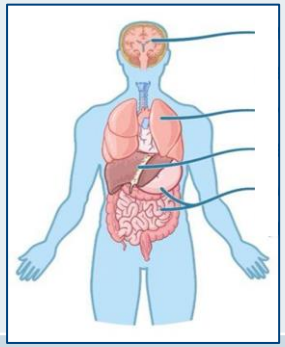
Impact of HCMV on transplant outcomes

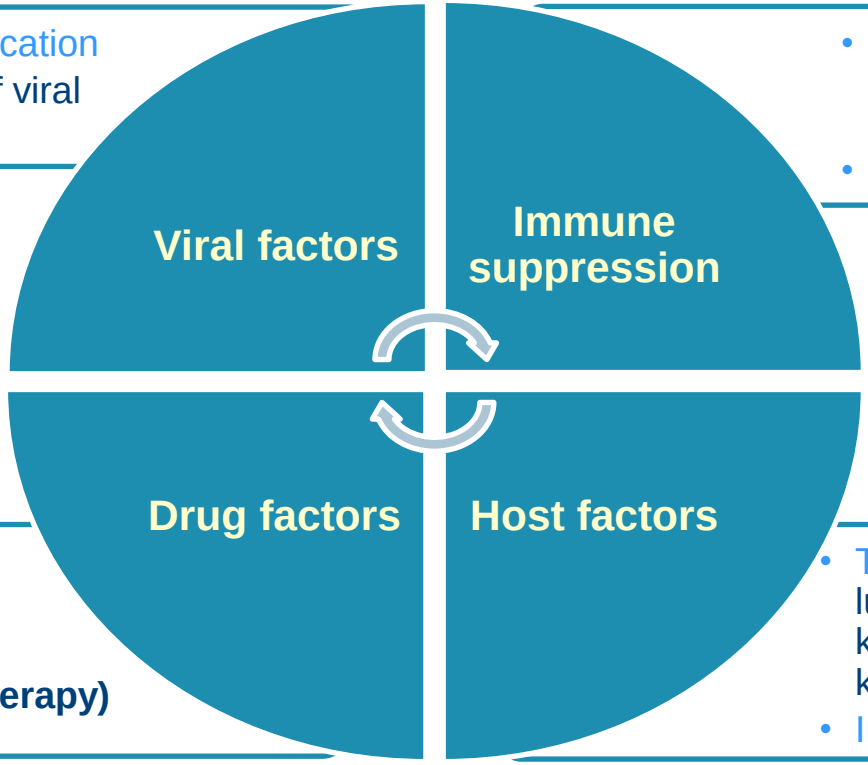


Direct effects
CMV syndrome
Tissue-invasive CMV disease - Gastrointestinal disease - Pneumonitis - Hepatitis - CNS disease - Retinitis - Nephritis - Pancreatitis - Myocarditis
Mortality



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 miellitus
Malignancies
Thrombosis
Mortality

Risk factors for HCMV drug-resistance



- High-level viral replication
- Multiple episodes of viral disease

- Potent immune suppression regimens (dose, duration, and overall intensity of drugs)
- T-cell depletion

- Suboptimal antiviral drug levels
- Prolonged antiviral drug administration

(prophylaxis > pre-emptive therapy)

- Type of transplant: > HSCT, lung/small intestine > pancreas, kidney-pancreas, heart > liver, kidney
- Immune serostatus

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

- *Effective in preventing **end organ disease (EOD)***
- *Risk of **late-onset CMV disease** once prophylaxis is stopped & of emergence of **drug-resistant CMV strains***

No universal agreement among transplant centers regarding:

Prophylaxis *versus* pre-emptive therapy

- **Prophylaxis:** an ant… …(as soon as the patient can tolerate oral medication) for a… …days 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)	1st line	approved		resistance	resistance		
HSV-2 (HHV-2)	1st line	approved		resistance	resistance		
VZV (HHV-3)	1st line	approved		resistance	resistance		
EBV (HHV-4)			off-label	off-label	off-label		
HCMV (HHV-5)			1st 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		

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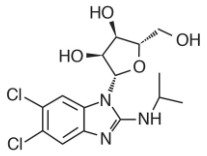
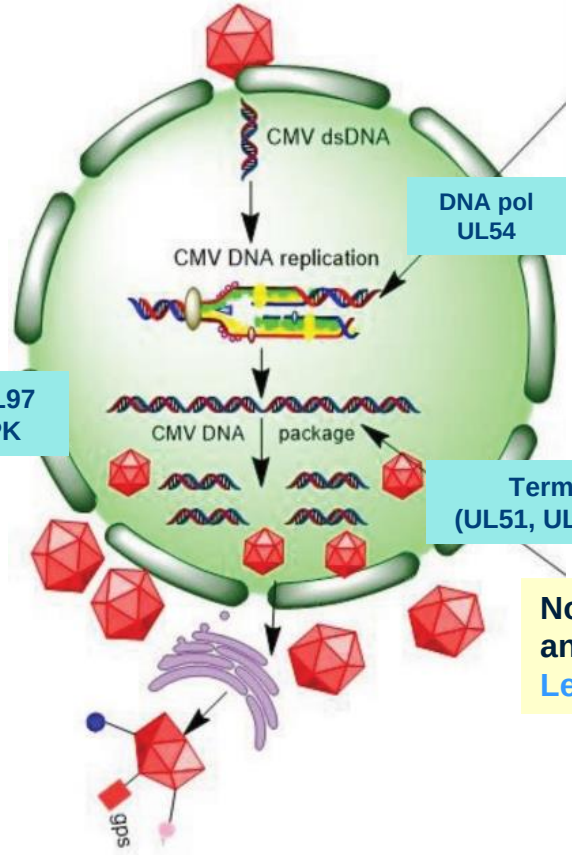
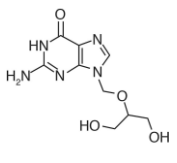
Anti-HCMV drugs

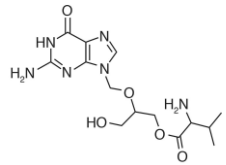


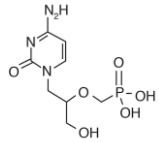
Benzimidazole riboside
Maribavir



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


Ganciclovir


Valganciclovir


Cidofovir


Foscarnet

DNA pol UL54

Viral genome replication

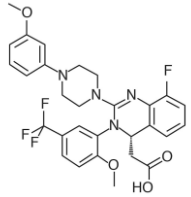
DNA elongation
DNA packaging
Capsid egress

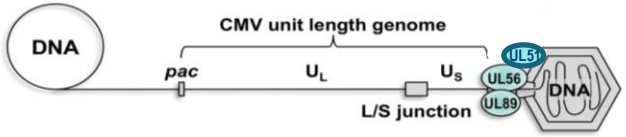
UL97 PK

Terminase (UL51, UL56, UL89)

Cleavage of viral DNA into unit length genomes

Non-nucleoside analogue
Letermovir





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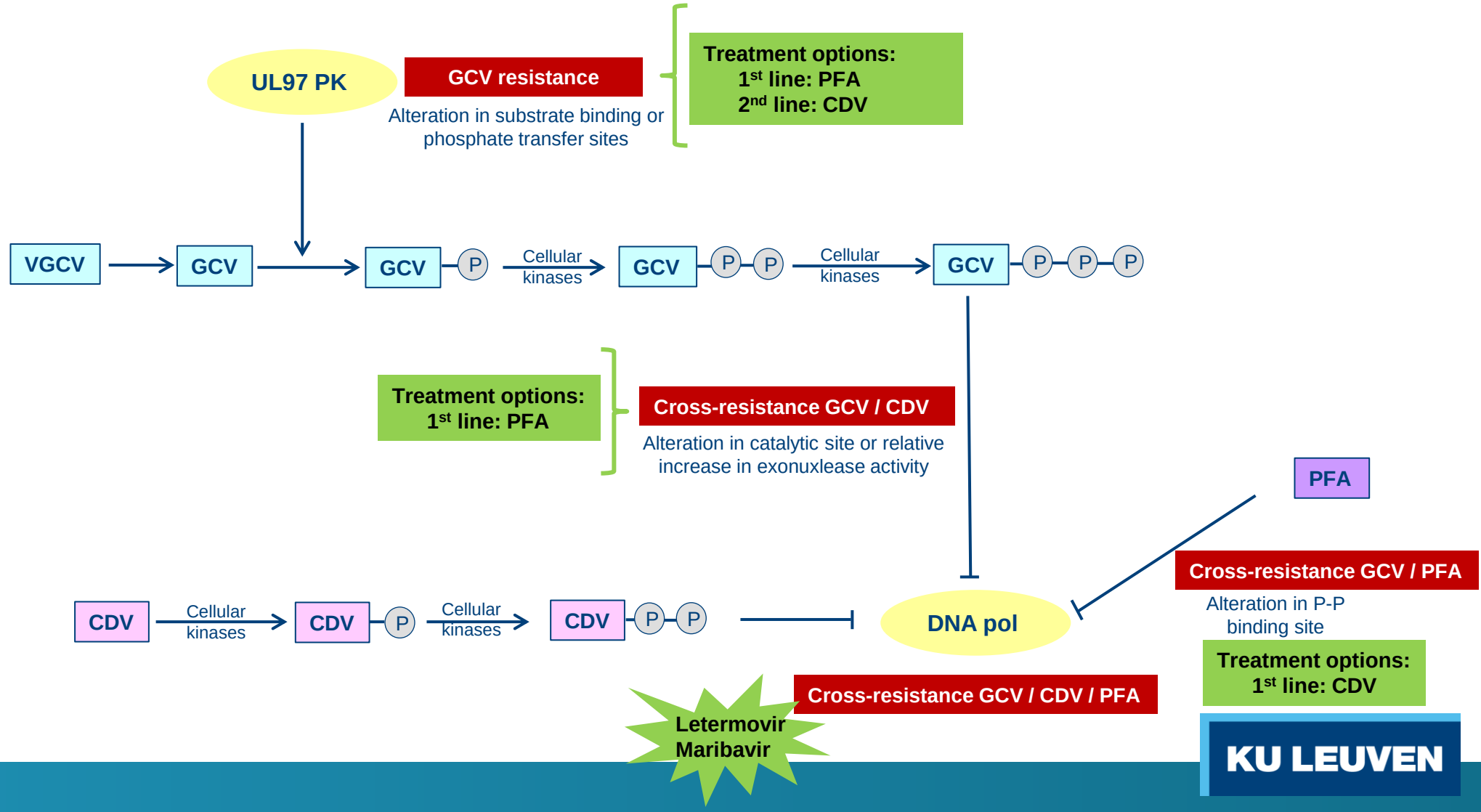
Mechanisms of drug-resistance in HCMV



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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 ≠ virological drug-resistance

Fundamental to evaluate virological drug-resistance

- **Riks 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 amplicifaction)
  - **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.

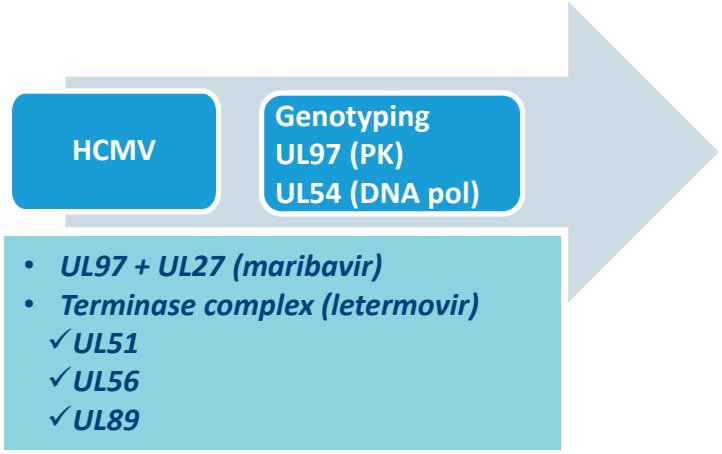
- There is a **restricted number of active antivirals** against herpesviruses and a limited number of viral targets.

  → limited options for alternative treatments in case of emergence of resistant viruses in IC patients.

  → most anti-HCMV are asssociated 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



1. Sample registration
2. DNA extraction using commercially available tests
3. PCR amplification
4. Purification of PCR products
5. Sanger (fluorescent dideoxy) sequencing involving capillary electrophoresis (ABI3730 DNA sequencer) & an automated base calling system (SeqScape).

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

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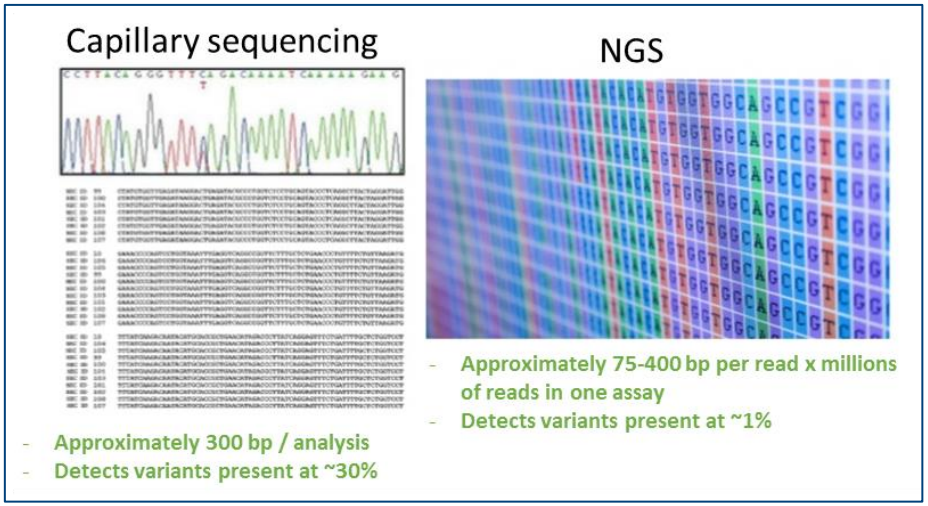
HCMV antiviral resistance tests available at RegaVir

- **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)**

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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 IU/mL = 1.72 copies/mL (range of the assay is 150- 6,000,000 IU/ml or 2.18-6.78 log 10 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 l**ocalized 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

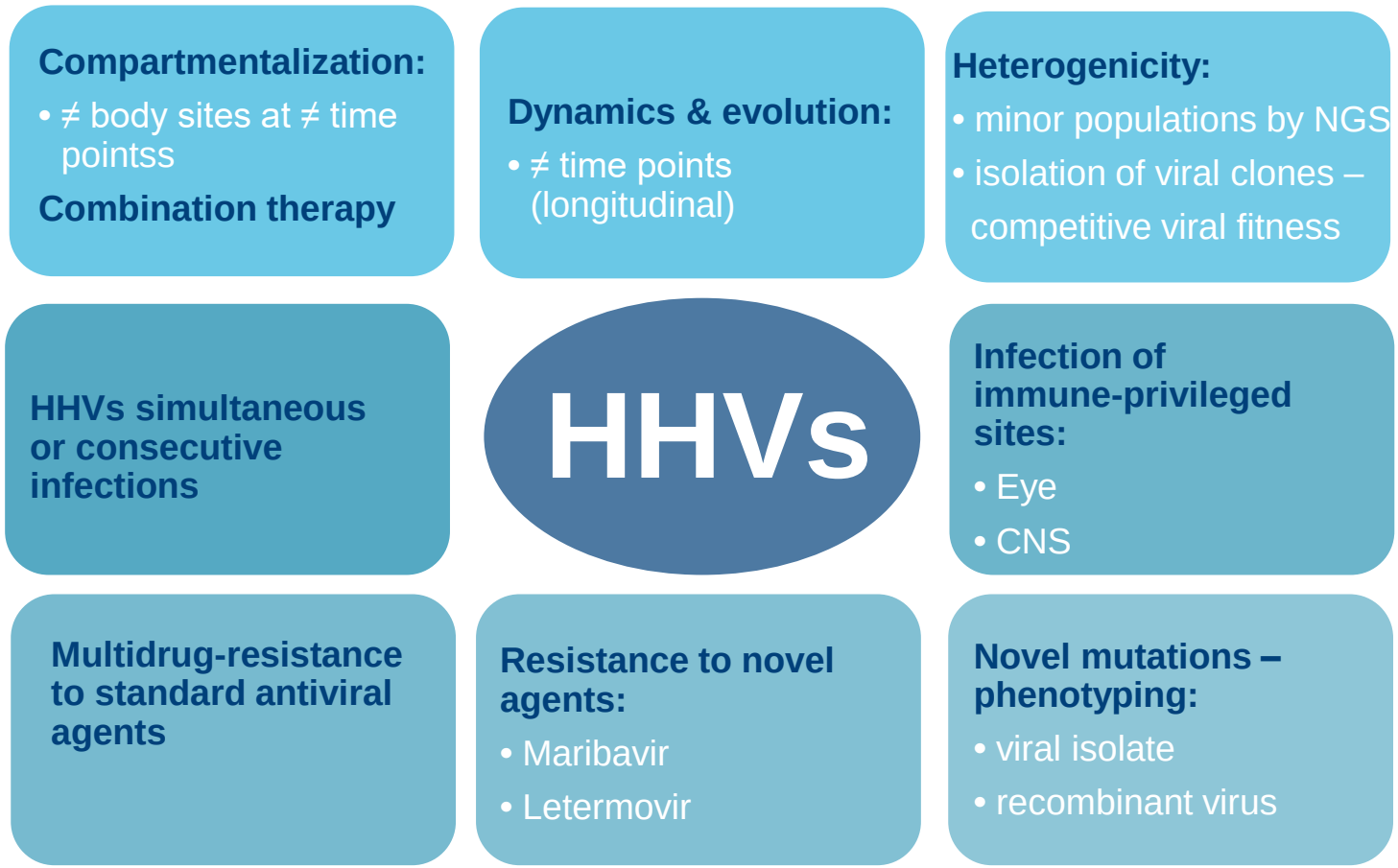
Instructions for shipping samples

- **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



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