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RegaVir platform: Case discussions antiviral resistance testing

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Leuven, October 25, 2022

Herpes simplex virus infections

HSV-1: Orofacial

Global prevalence: 67%

3.7 billion people under age 50

- **Mainly transmitted by oral-to-oral contact**
- **Causes cold sores, but it can also lead to genital herpes**
- Rare complications include encephalitis (brain infection) or keratitis (eye infection).

HSV-2: Anogenital

Global prevalence: 13%

491 million people under aged 50

- **Mainly transmitted sexually**
- **Causes mainly genital herpes**
- Rare complications of HSV-2 include meningoencephalitis (brain infection) and disseminated infection.

- **Neonatal herpes** occur when an infant is exposed to HSV during delivery.
- Rare, occurring in an estimated 10 out of every 100 000 births globally.
- However, it is a serious condition that can lead to lasting neurologic disability or death.
- Risk for neonatal herpes is greatest when a mother acquires HSV for the first time in late pregnancy.

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Herpes simplex virus infections

- Most HSV infections are asymptomatic, but symptoms of herpes include painful blisters or ulcers that can recur over time.
- Infection with HSV-2 increases the risk of acquiring and transmitting HIV infection.

- Reactivation in ~40% of adults
- Up to 80% reactivation in HSCT recipients

- **Immunocompromised individuals:**
  - **More invasive disease**
  - **Slower healing**
  - **Prolonged viral shedding**
  - **Risk of dissemination**

Clinical syndromes associated with human herpesviruses

	HSV-1	HSV-2	VZV	CMV	EBV	HHV-6	HHV-7	KSHV
Gingivostomatitis	+	+	-	-	-	-	-	-
Genital lesions	+	+	-	-	-	-	-	-
Keratoconjuntivitis	+	+	+	-	-	-	-	-
Cutaneous lesions	+	+	+	-	-	-	-	+
Neonatal infection	+	+	+	+	-	-	-	-
Retinitis	+	+	+	+	-	-	-	-
Esophagitis	+	+	+	+	-	-	-	-
Pneumonitis	+	+	+	+	+	+	-	-
Hepatitis	+	+	+	+	+	+	-	-
Meningitis	-	+	+	-	-	+	-	-
Encephalitis	+	+	+	+	+	+	-	-
Myelitis	+	+	+	+	+	+	-	-
Mononucleosis	-	-	-	+	+	+	-	+?
Hemolytic anemia	-	-	+	+	+	-	-	-
Leukopenia	-	-	+	+	+	+	-	-
Trombocytopenia	-	-	+	+	+	+	-	-

Classical antiviral agents for the management of HHV infections

Target	Anti-herpesvirus drug	Trade name	Indication	Route of administration
DNA pol	**Acyclovir**	Zovirax®	**HSV-1, HSV-2**, VZV	Oral, iv, topical
DNA pol	**Valacyclovir (VACV)**	Valtrex®, Zelitrex®	**HSV-1, HSV-2**, VZV	Oral
DNA pol	**Penciclovir (PCV)**	Vectavir®, Denavir®	**HSV-1, HSV-2**, VZV	Topical
DNA pol	**Famciclovir (FAM)**	Famvir®	**HSV-1, HSV-2**, VZV	Oral
DNA pol	**Brivudine (BVDU)**	Zostex®, Zerpex®	**HSV-1**, VZV	Oral
DNA pol	Ganciclovir (GCV)	Cytovene® Cymevene®	HCMV	Oral, iv, intravitreal
DNA pol	Valganciclovir (VGCV)	Valacyte®	HCMV	Oral
DNA pol	Cidofovir	Vistide®	HCMV	iv
DNA pol	Foscarnet (PFA)	Foscavir®	HCMV, HSV-1, HSV-2, VZV	iv
Terminase	Letermovir (LMV)	Prevymis®	HCMV	Oral

Other antiviral agents for the management of HSV infections

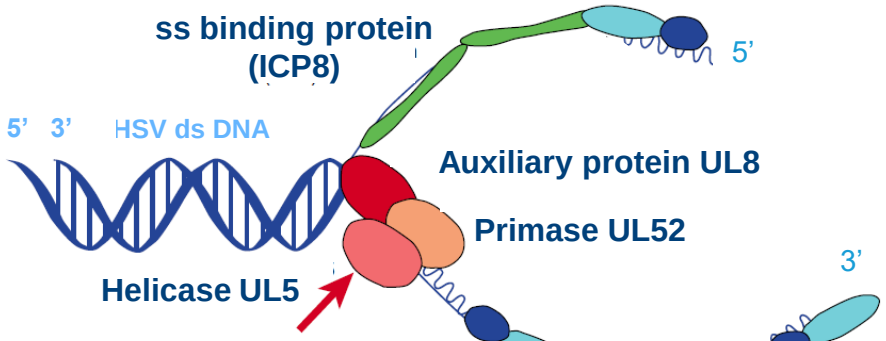
Target	Anti-herpesvirus drug	Trade name	Indication	Route of administration
DNA polymerase	**Trifluridine (trifluorothymidine)**	Viroptic®	Herpes simplex keratoconjunctivitis	Ophthalmic drops
DNA polymerase	**Vidarabine (Ara-A)**	VIRA-A®	Acute keratoconjuntivitis & recurrent epithelial keratitis	Ophthalmic ointment
Helicase-primase complex	**Pritelivir**	Investigational	HSV-1, HSV-2, VZV	Oral

Viral DNA replication: target for antiviral drugs

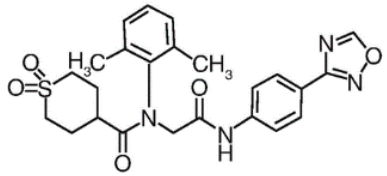
HSV / VZV

Nucleoside analogues
(ACV, VACV, PCV, FCV)
Nucleotide analogues
(CDV)
Pyrophosphate analogues
(PFA)

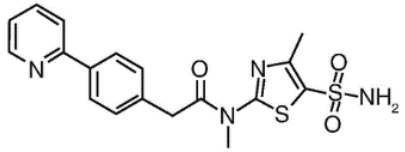
DNA polymerase
(UL30 and UL42)



Helicase-primase
HPIs stabilize the helicase-primase complex at replication fork



Amenamevir (ASP2151)
Astellas Pharma, Maruho



Pritelivir (AIC316, Bay 57-1293)
Aicuris

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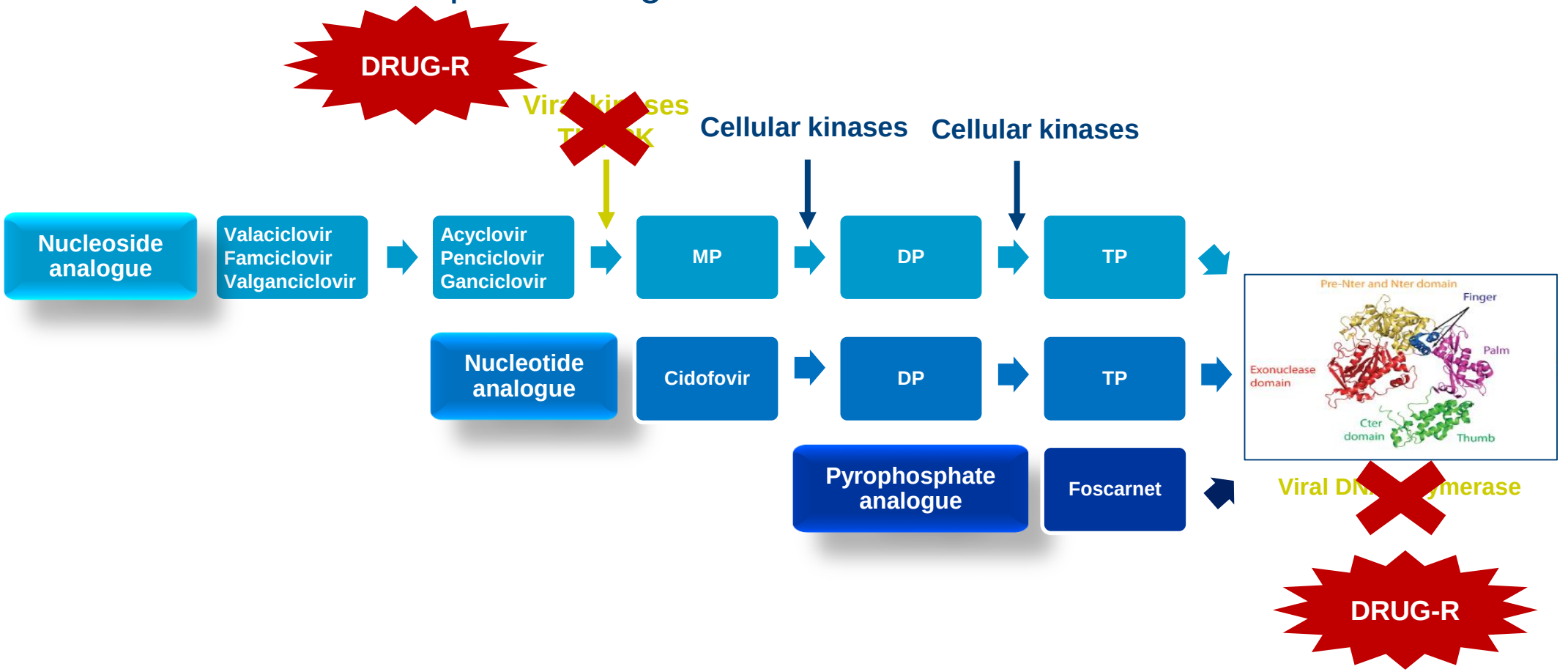
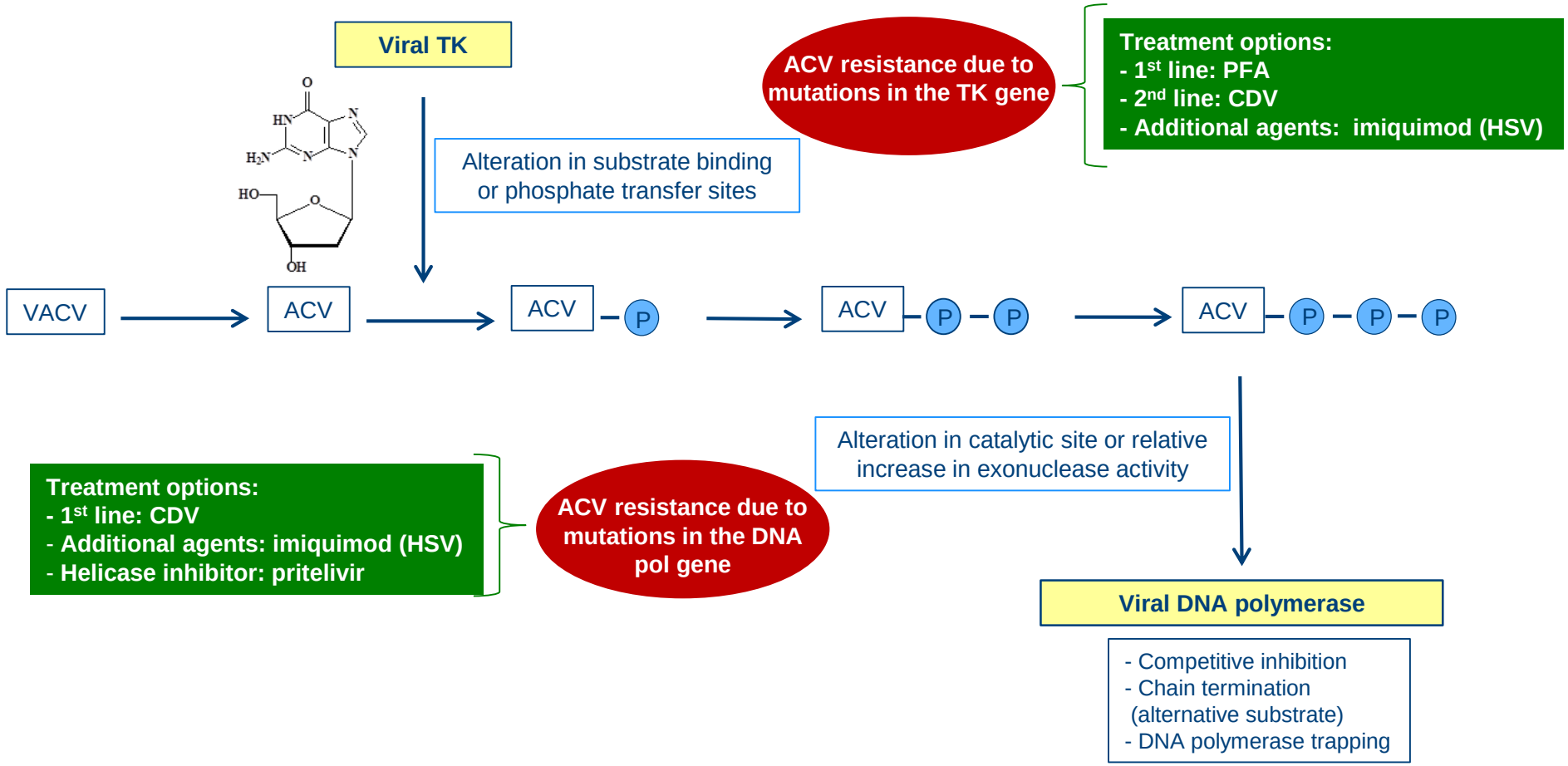
DNA polymerase: important antiviral target

- Classical anti-herpesvirus agents



Mechanisms of action of ACV against HSV & VZV



Acyclovir resistance (ACV-R)

Risk factors for resistance emergence

- Prolonged ACV use
- Suboptimal ACV dosing
- (degree of) immunosuppression
- Ongoing viral replication

- Infections with HSV occur most commonly in the **first month after transplantation**

  → **ACV prophylaxis for at least 1 month after SOT** is recommended

Prevalence of ACV-R HSV infections – Systematic review

Concept 1: herpes simplex (57.250 results)

"Herpes Simplex"[Mesh] OR herpes-simplex*[tiab] OR "Herpesvirus 1, Human"[Mesh] OR "human Herpesvirus 1"[tiab] OR "Herpesvirus 2, Human"[Mesh] OR "Human Herpesvirus 2"[tiab] OR HSV[tiab] OR HHV-1[tiab] OR HHV-2[tiab] OR “herpes labialis"[tiab] OR “labial herpes”[tiab] OR “herpes facialis”[tiab] OR “herpes genitalis”[tiab] OR “genital herpes”[tiab] OR herpetic-keratiti*[tiab] OR herpes-keratiti*[tiab] OR Herpetic-Stomatiti*[tiab] OR “herpetic gingivostomatitis”[tiab] OR "Encephalitis, Herpes Simplex"[Mesh] OR herpes-simplex-encephaliti*[tiab] OR herpes-encephaliti*[tiab] OR herpetic-encephaliti*[tiab] OR herpetic-meningoencephaliti*[tiab]

Concept 2: antiviral therapy (171.563 results)

"Antiviral Agents"[Mesh:NoExp] OR antiviral*[tiab] OR anti-viral*[tiab] OR nucleoside-analog*[tiab] OR "Acyclovir"[Mesh] OR “acyclovir”[tiab] OR “aciclovir”[tiab] OR “ACV”[tiab] OR “valacyclovir”[tiab] OR “valaciclovir”[tiab]

Concept 3: drug resistance or drug susceptibility (2.750.774 results)

"Drug Resistance, Viral"[Mesh] OR "Drug Resistance, Microbial"[Mesh:NoExp] OR drug-resistan*[tiab] OR antiviral-resistan*[tiab] OR antiviral-drug-resistan*[tiab] OR “resistant”[tiab] OR resistance*[tiab] OR "sensitive”[tiab] OR sensitivit*[tiab] OR "susceptible"[tiab] OR susceptibilit*[tiab]

Search: #1 AND #2 AND #3 (3202 results)

Schalkwijk HH et al, Biochemical Pharmacology, in press

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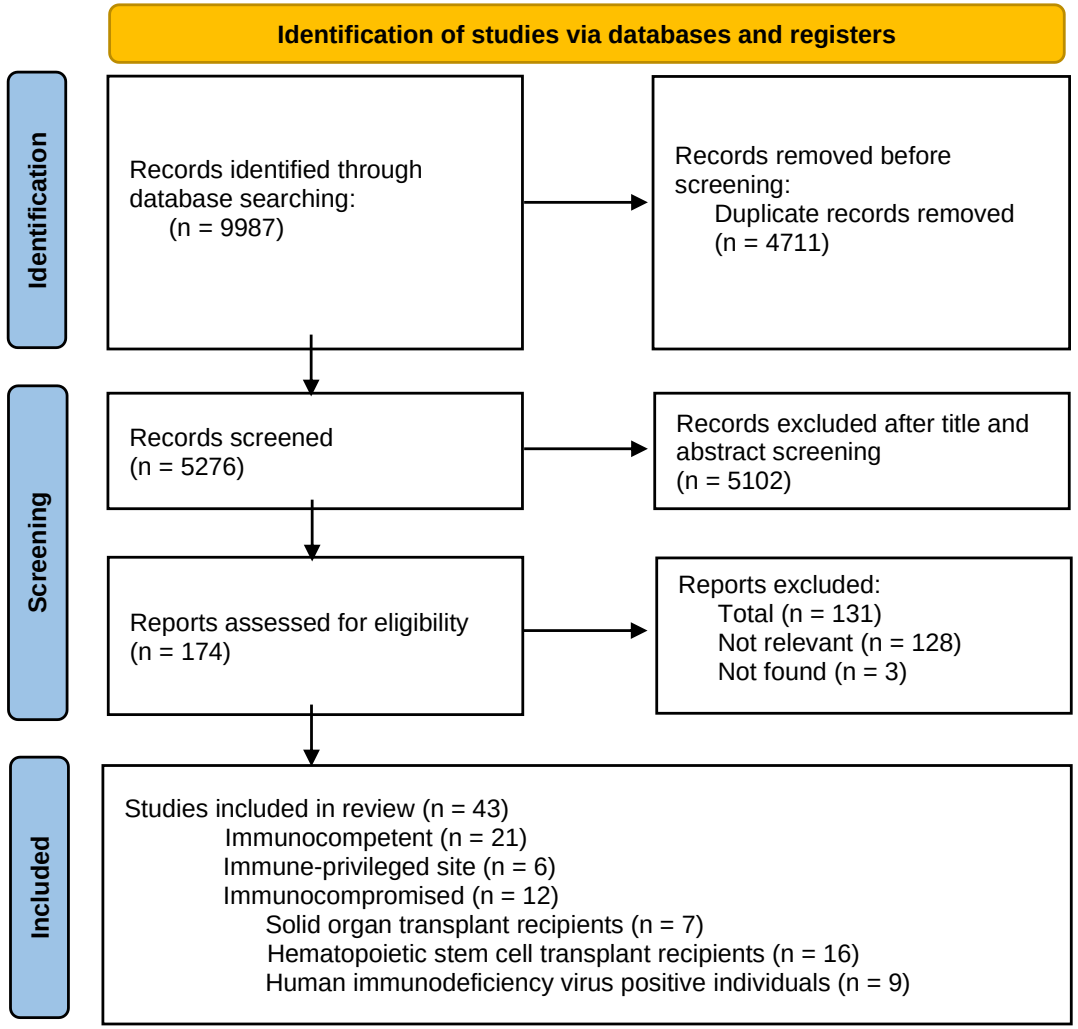
Prevalence of ACV-R HSV infections



Exclusion criteria:

- Written in foreign language
- < 5 patients
- Clinical trial
- Patient selection based on suspected resistance
- Resistance testing not performed
  - ✓ phenotypical
  - ✓ genotypical

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Prevalence of ACV-R HSV infections

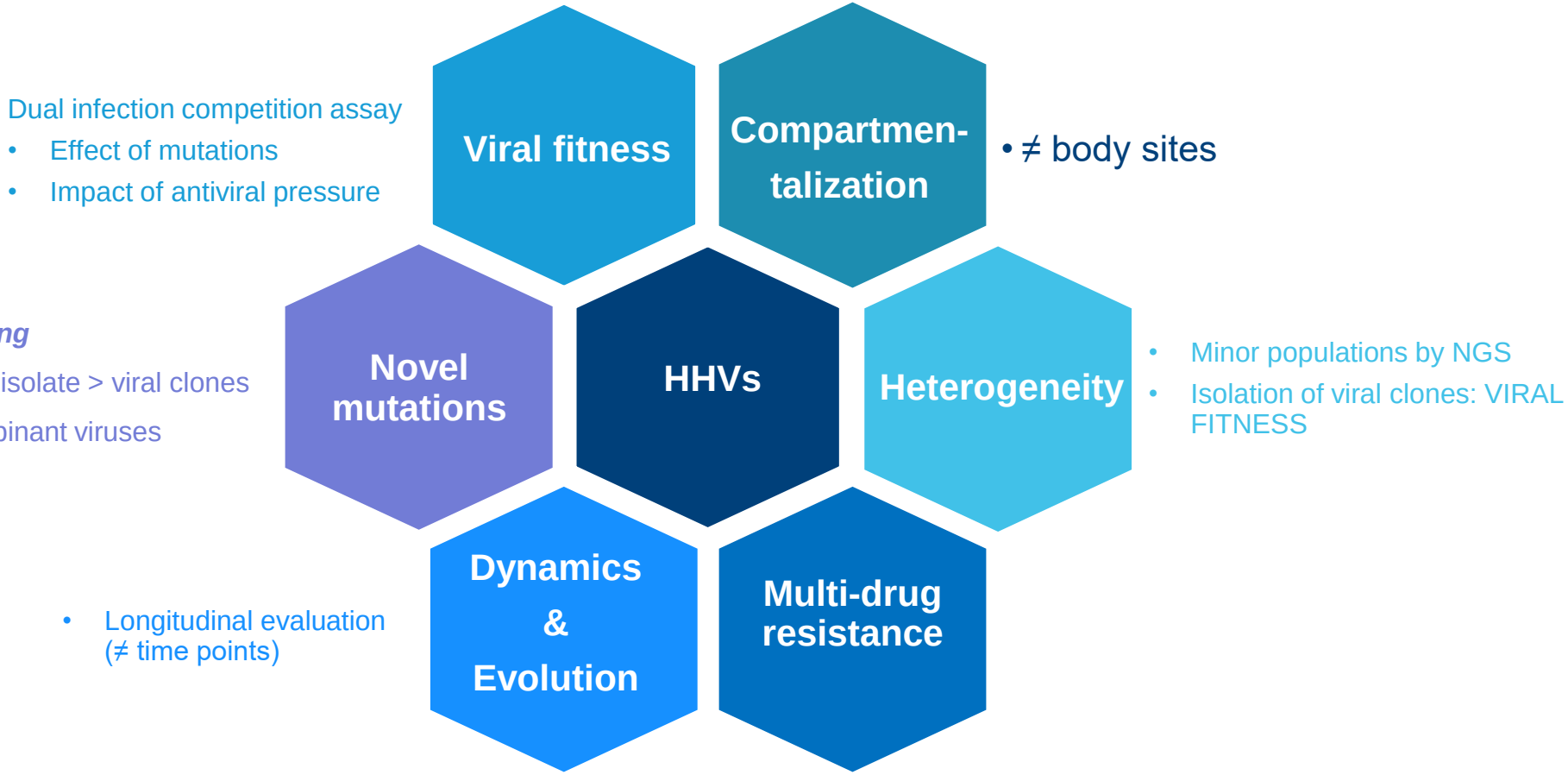
Immunocompetent (21 studies)		**< 1% (0% - 6.2%)**
Immune privileged sites (6 studies)	Eye infections	0% - 34.6%
	Herpetic keratitis	6.4% - 34.6%
	CNS	0% (1 study)
Immunocompromised (13 studies)		**> 3%** (0% - 28.8%)
	SOT (7 studies)	**< 3.5%** (0% -10%) (> lung & heart Tx)
	HIV infected (9 studies)	**3.4 – 7.3%** (0% - 25%) HSV-2 > HSV-1
	HSCT (16 studies)	**0% - 100%** HSV-1 > HSV-2

Prevalence of ACV-R HSV infections

Variability in phenotyping results

- assay system
- cell line
- viral inoculum concentration
- range antiviral concentrations tested
- cut-off resistance

Case study I: A 17-years old male SCID / HSCT recipient with recurrent orofacial and genital HSV-1 lesions



Herpesvirus genotyping

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

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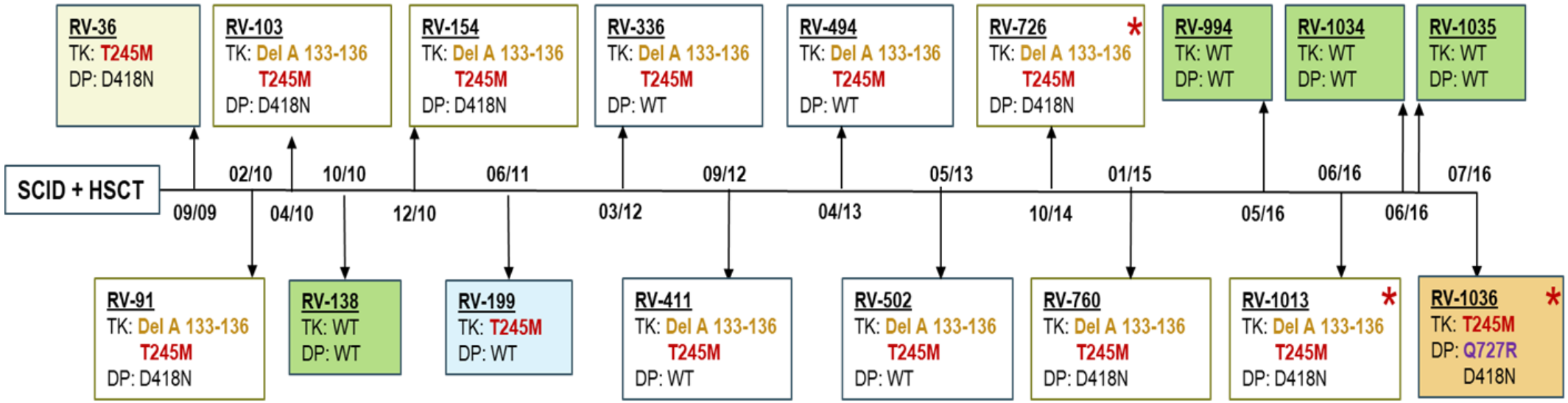
Case study I: A 17-years old male SCID / HSCT recipient with recurrent orofacial and genital HSV-1 lesions



*genital lesion

Dynamics & Evolution

- 17 time points (7 years)
- TK substitution and additional TK deletion
- Acquisition of PFA resistance
- Spreading of the infection

Compartmentalization

- Orofacial & Anogenital lesions

Novel mutations

- DP D418N
- DP Q727R

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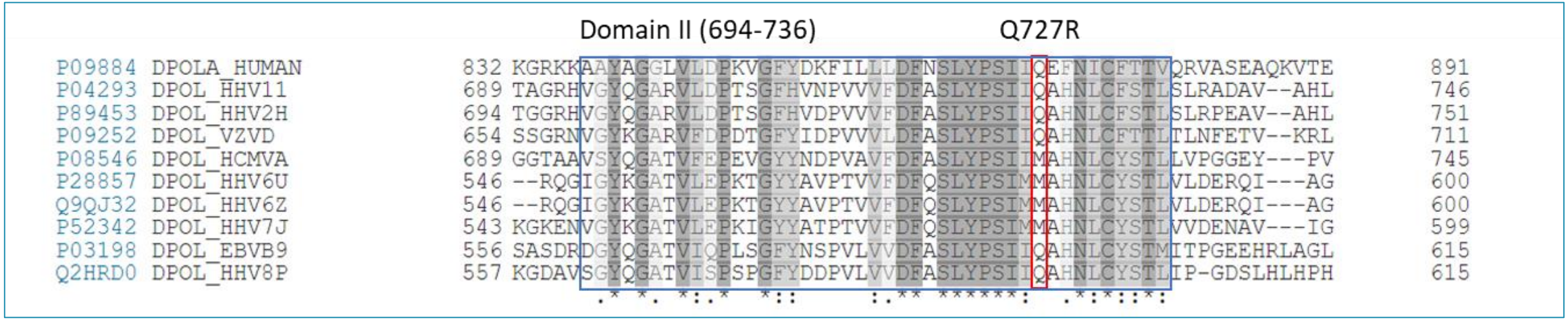
Case study I: A 17-years old male SCID / HSCT recipient with recurrent orofacial and genital HSV-1 lesions

- **Investigate previously undescribed mutations in HSV-1 DNA pol**

D418N: - unknown polymorphism
- no effect on drug susceptibility

Q727R: - resistance to nucleoside, nucleotide, and pyrophosphate analogues
- homologous changes in **HSV-2 (Q732R)** and in **VZV (Q692R)** confer drug resistance



Case study I: A 17-years old male SCID / HSCT recipient with recurrent orofacial and genital HSV-1 lesions

- Heterogeneity of viral isolates by next-generation sequencing

	Frequency %																
	RV-36	RV-91	RV-103	RV-138	RV-154	RV-199	RV-336	RV-411	RV-494	RV-502	RV-726	RV-760	RV-994	RV-1013	RV-1034	RV-1035	RV-1036
	O.F. 10/09	O.F. 02/10	O.F. 04/10	O.F. 10/10	O.F. 12/10	O.F. 06/11	O.F. 04/12	O.F. 09/12	O.F. 06/13	O.F. 04/13	Genital 10/14	O.F. 01/15	Gargle 04/16	Genital 06/16	Gargle 06/16	Gargle 06/16	Genital 07/16
TK del A 133-136	-	93.9	94.9	-	94.0	3.5	90.5	90.2	93.6	94.0	95.4	93.9	-	93.4	-	-	-
TK T245M	99.9	99.9	99.9	-	100	99.9	99.9	99.9	99.8	99.9	100	99.9	-	99.9	-	-	99.5
DP D418N	99.9	99.9	99.9	-	99.9	-	-	-	-	-	95.4	99.9	-	99.9	-	-	99.9
DP Q727R	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	99.7

No heterogeneity

- Orofacial & genital lesions have same genetic background and resistance mutations → **self-infection**
  TK: G6C, P42L, R89Q, C251G, S321P & DNA pol: T566A, K700R
- Heterogeneity in 11 out of 17 isolates (not detected by Sanger)
- 1 sample showed heterogeneity in DP

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Case study I: A 17-years old male SCID / HSCT recipient with recurrent orofacial and genital HSV-1 lesions

- Heterogeneity of viral isolates – isolation of plaque purified viruses (viral clones)

Isolate	N° of clones	Mutations in viral TK	Mutations in viral DP
RV-36	3	T245M	D418N
RV-138	3	-	-
RV-199	2	Del A nts133-136 T245M	-
	1	T245M	-
RV-726*	3	Del A nts133-136 T245M	D418N
RV-1013*	3	Del A nts133-136 T245M	D418N
RV-1035	1	-	R264C
	2	-	-
RV-1036*	5	T245M	D418N, **Q727R**

* Genital lesions

- **7 distinct types of viral clones**
  wild-type
  TK mutation
  DP mutation
  TK/DP double mutant

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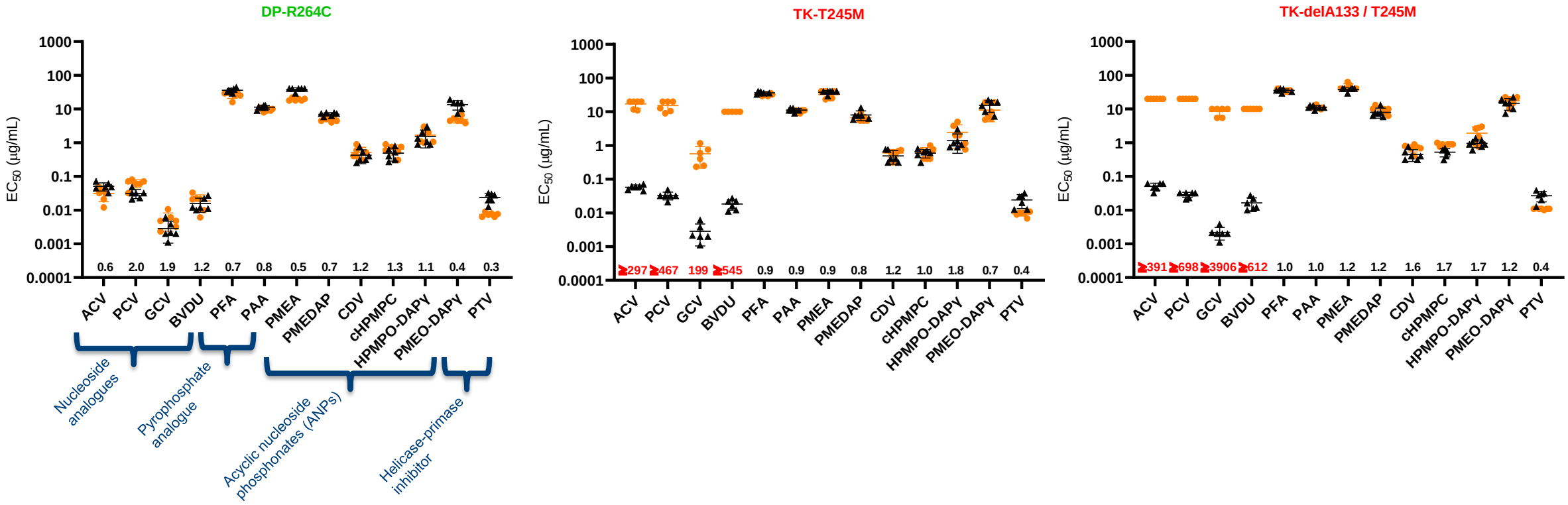
Case study I: A 17-years old male SCID / HSCT recipient with recurrent orofacial and genital HSV-1 lesions

- Susceptibility profile of viral clones



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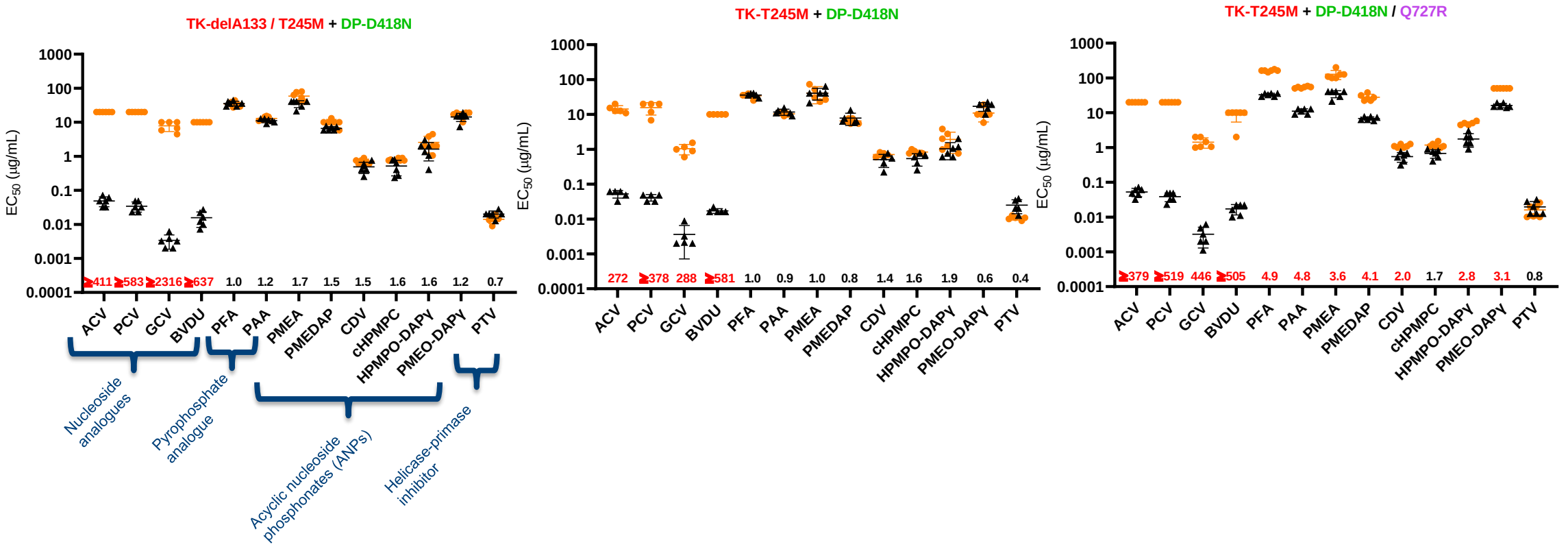

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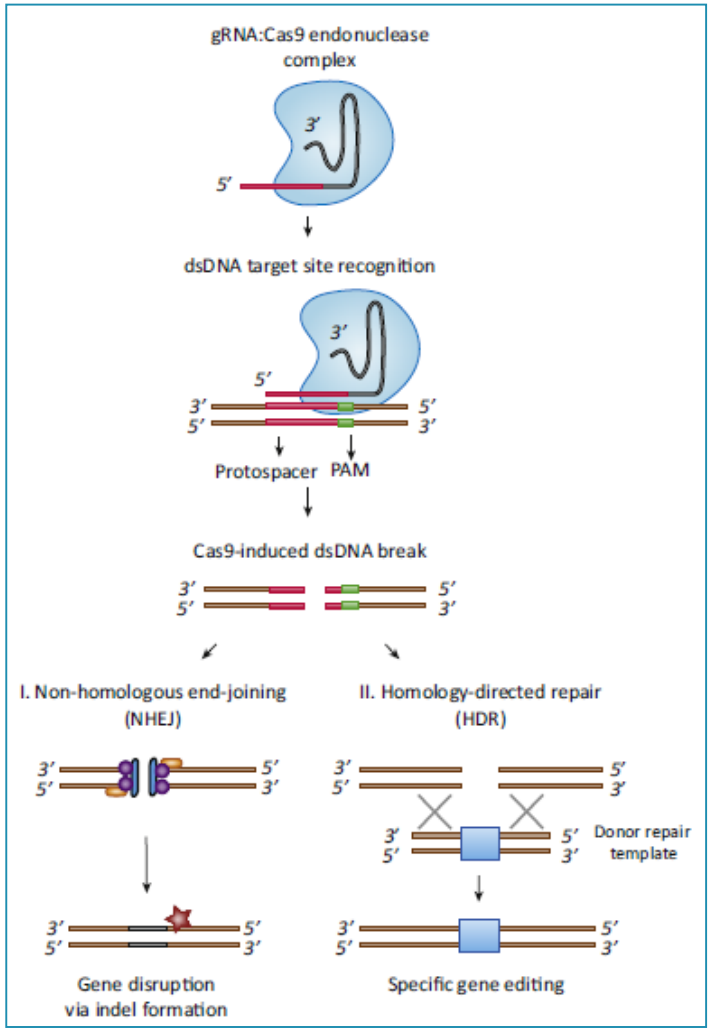
Case study I: Validation of DNA pol Q727R substitution by CRISPR-Cas9 genome editing

Transfect HEK293T cells
- Plasmid with Cas9 + gRNA
- ssDNA template

Infect transfected HEK293T cells
- Different concentration of wild-type virus

Obtain viral clones
- Harvest virus from supernatant
- Plaque purification

Determination of viral variants
- DNA extraction
- Sanger Sequencing
- Antiviral Assays



Soppe and Lebbink. Trends Microbiol. 2017

- The 20nt **single-guide RNA (sgRNA)** guides the **Cas9 nuclease** to the complementary genomic target locus, which must be located next to the **protospacer adjacent motif (PAM) (5'-NGG-3')**.
- The **protospacer adjacent motif (PAM) i**s a short DNA sequence (usually 2-6 base pairs in length) that is recognized by Cas9 nuclease and is generally found 3-4 nucleotides downstream from the cut site.
- Cas9 unwinds the DNA duplex and cleaves both strands to form a double-strand break (DSB). DSBs can be repaired by non-homologous end-joining (NHEJ) or homology-directed repair (HDR) using a donor DNA template [i.e. single-stranded oligo DNA nucleotides (ssODNs)].
- While NHEJ is used for disruption of a target gene by generating premature stop codons or frameshifts, HDR is used to introduce single-base substitutions.

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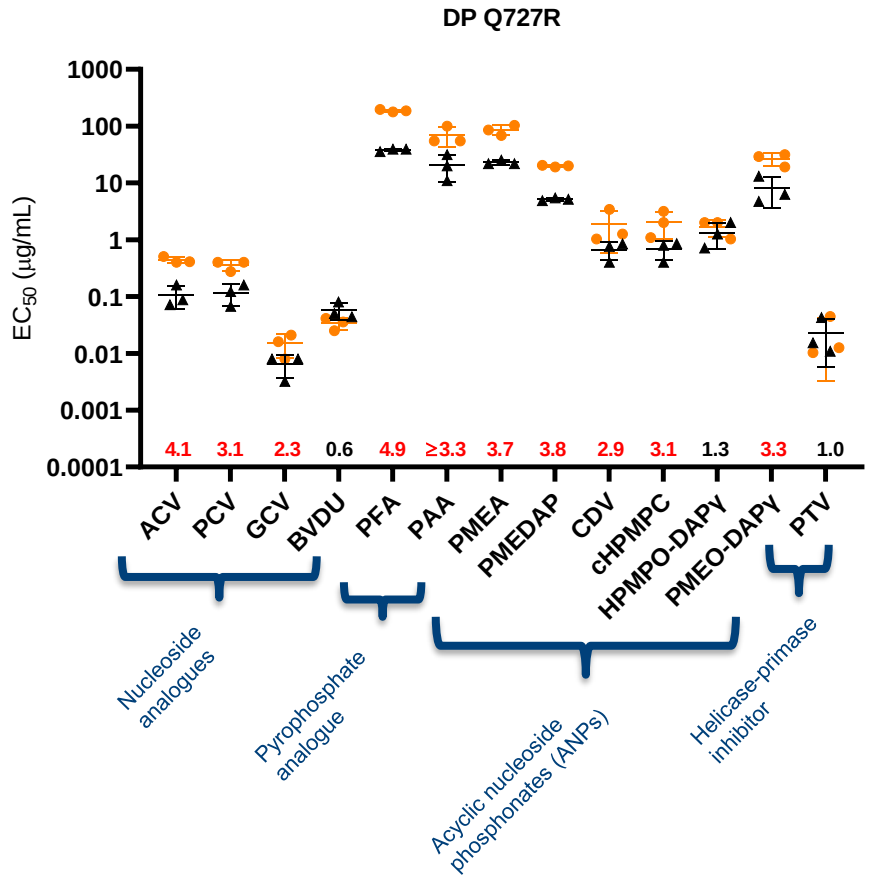

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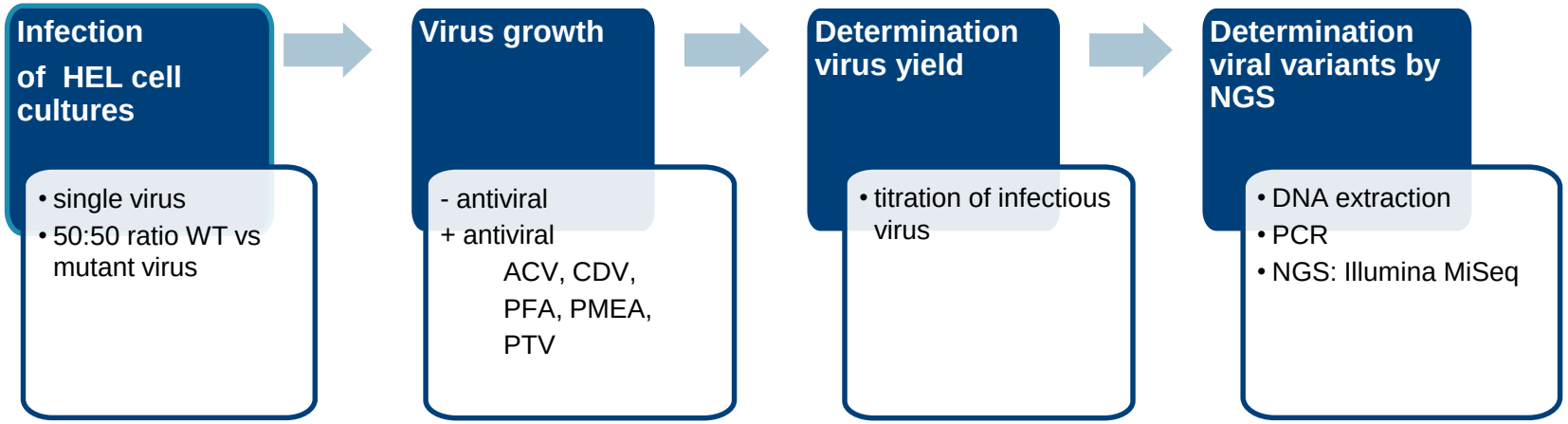
Case study I: Viral replication fitness of the DNA pol Q727R mutant

- Dual infection competition assays



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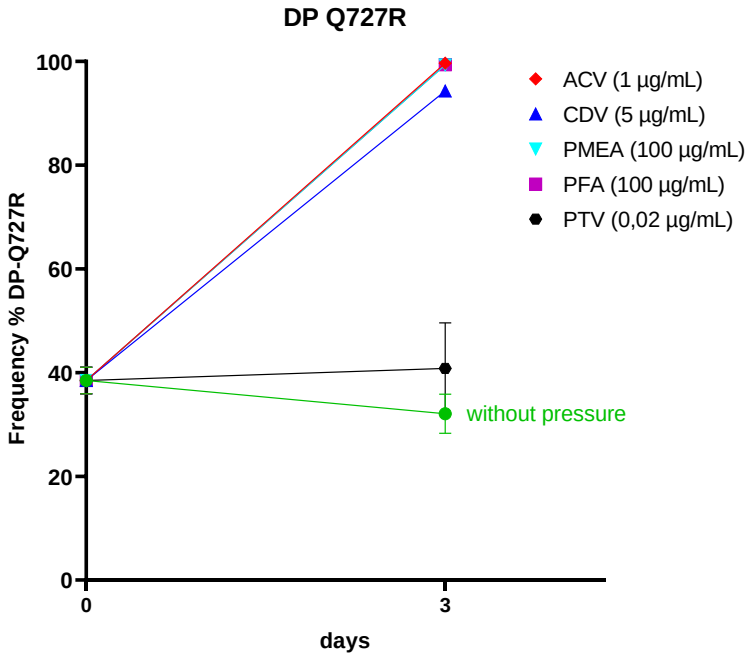

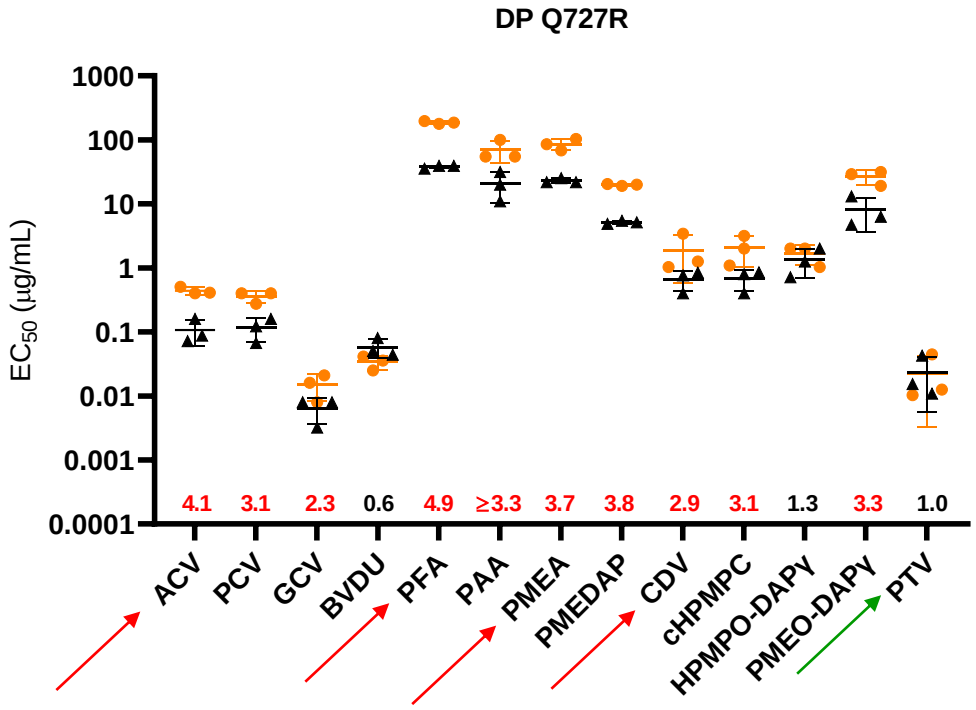

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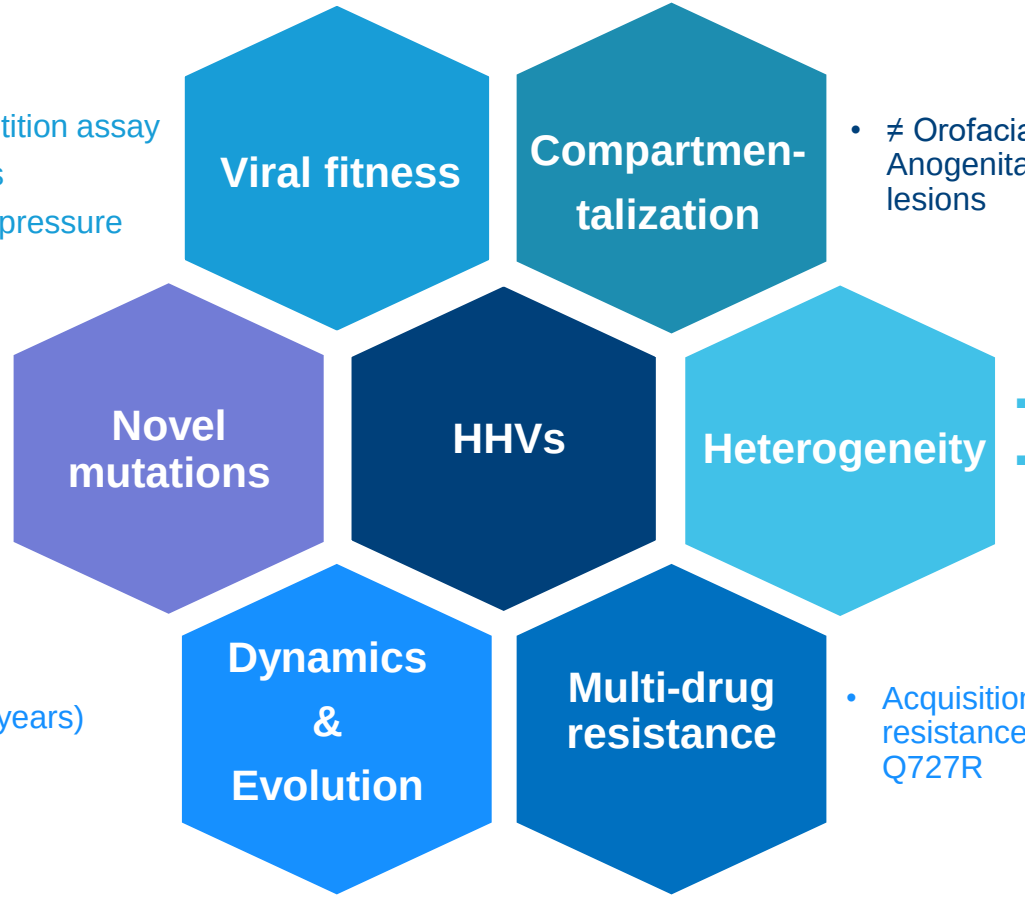
Case study I: A 17-years old male SCID / HSCT recipient with recurrent orofacial and genital HSV-1 lesions



Dual infection competition assay
- Effect of mutations
- Impact of antiviral pressure

- ≠ Orofacial vs Anogenital lesions

Phenotyping
- Clinical isolate > viral clones
- Implementation of CRISPR/Cas9 for validation novel mutations

- In 11 of 17 isolates (NGS)
- 7 distinct types of viral clones isolated

- 17 time points (7 years)

- Acquisition of multidrug resistance mutation DP Q727R

Herpesvirus genotyping

❖ **Prospectively:** capillary (Sanger sequencing)

❖ **Retrospectively:** next-generation sequencing (NGS)

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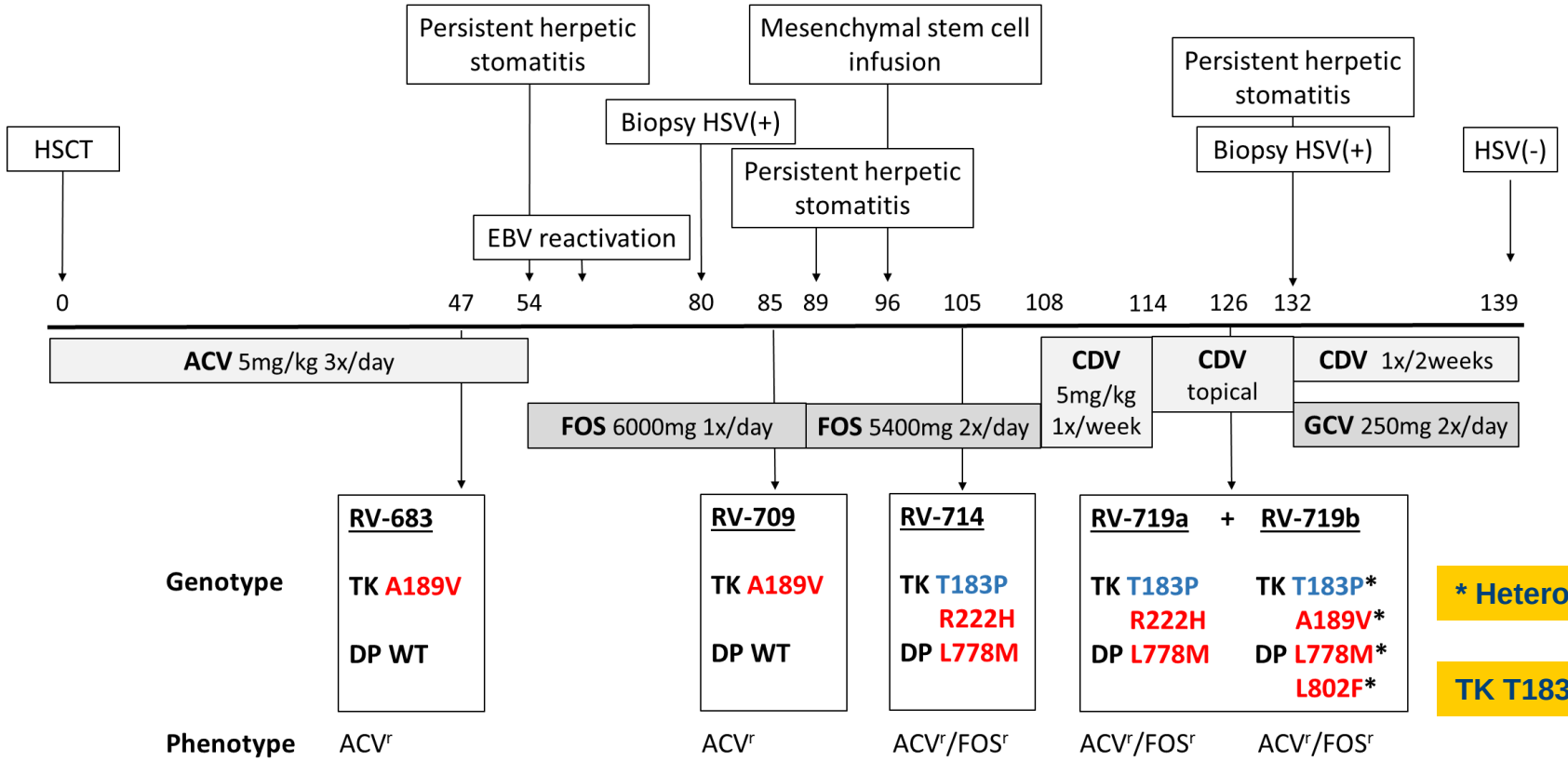
Case study II: a 41-year-old male with GATA-binding factor 2 (GATA2) deficiency / HSCT



*** Heterogeneity**

TK T183P: novel mutation

Compartmentalization

Schalkwijk HH et al, Journal of Antimicrobial Chemotherapy, dkac297, https://doi.org/10.1093/jac/dkac297

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Case study II: a 41-year-old male with GATA-binding factor 2 (GATA2) deficiency / HSCT

- Heterogeneity of viral isolates by next-generation sequencing (NGS)

Isolate	Day post-transplantation	Gene	Variant	Frequency (%)
RV683	47	TK	deletion G nt430-436#	1.0
			A189V	99.7
RV709	85	TK	T183P#	4.1
			A189V	95.2
		→	R222H#	4.2
		DP →	**L778M#**	4.7
RV714	105	TK	T183P	97.9
		→	R222H	99.8
		DP →	L778M	99.7
RV719a	126	TK	T183P	97.1
			R222H	95.9
		DP	L778M	98.5
RV719b	126	TK	T183P	33.2
			A189V	62.4
			R222H#	4.0
		DP	L778M	30.4
			L802F	62.4

 # not detected by Sanger sequencing.

Advantage of NGS to detect:
- **Minor viral populations**
- **Emergence of drug-resistance**

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Case study II: a 41-year-old male with GATA-binding factor 2 (GATA2) deficiency / HSCT

- Heterogeneity of viral isolates – isolation of plaque purified viruses (viral clones)

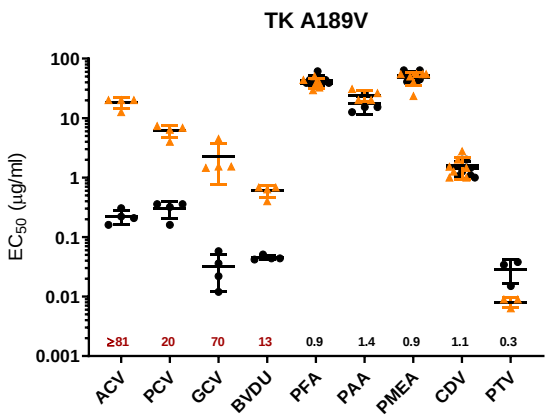
Isolate	Day post-transplantation	Number of clones	Mutations in viral TK	Mutations in viral DP
RV683	47	9	A189V	-
RV-709	85	5	A189V	-
		2	T183P, R222H	L778M
RV714	105	5	T183P, R222H	L778M
RV719a	126	5	T183P, R222H	L778M
RV719b	126	2	A189V	L778M
		1	T183P	L778M
		11	A189V	L802F
		1	T183P	L802F

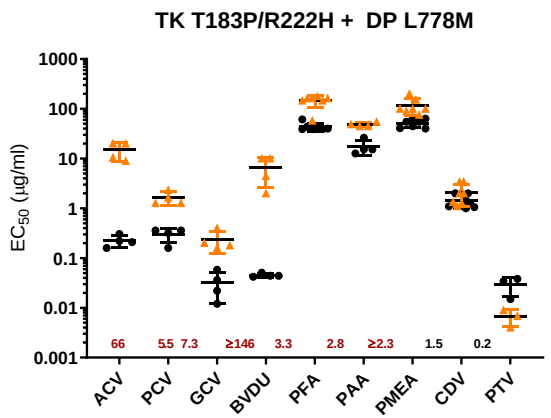
- no mutations detected

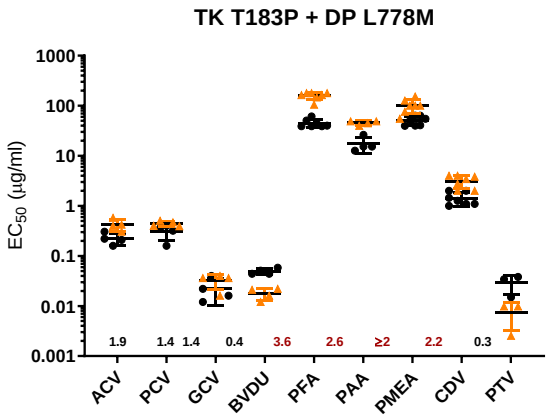
6 ≠ types of clones

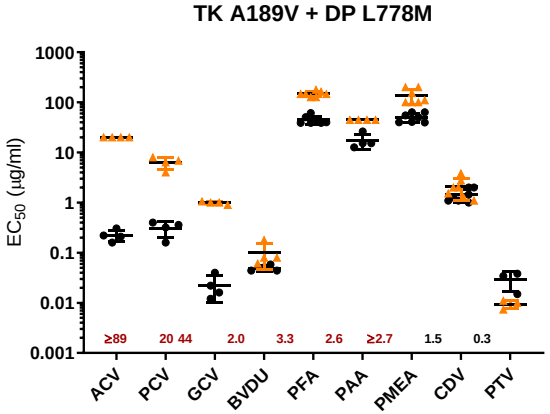
Case study II: a 41-year-old male with GATA-binding factor 2 (GATA2) deficiency / HSCT

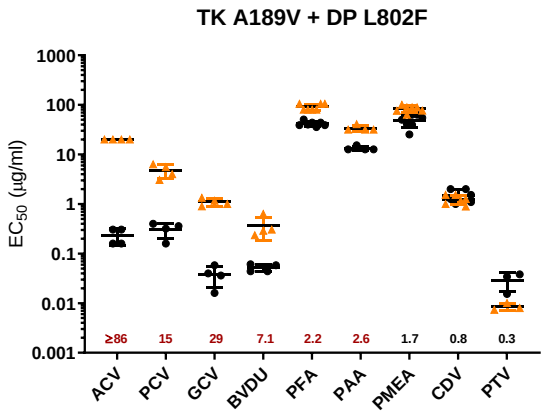
- Susceptibility profile of viral clones

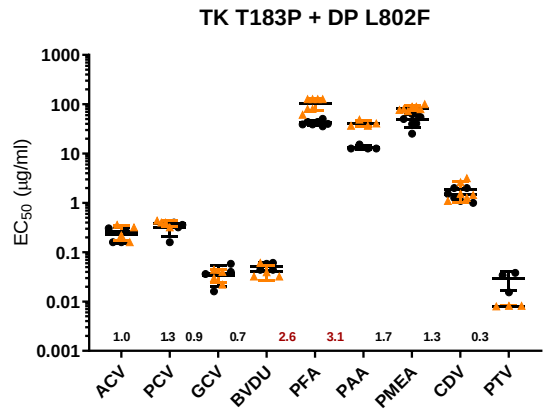












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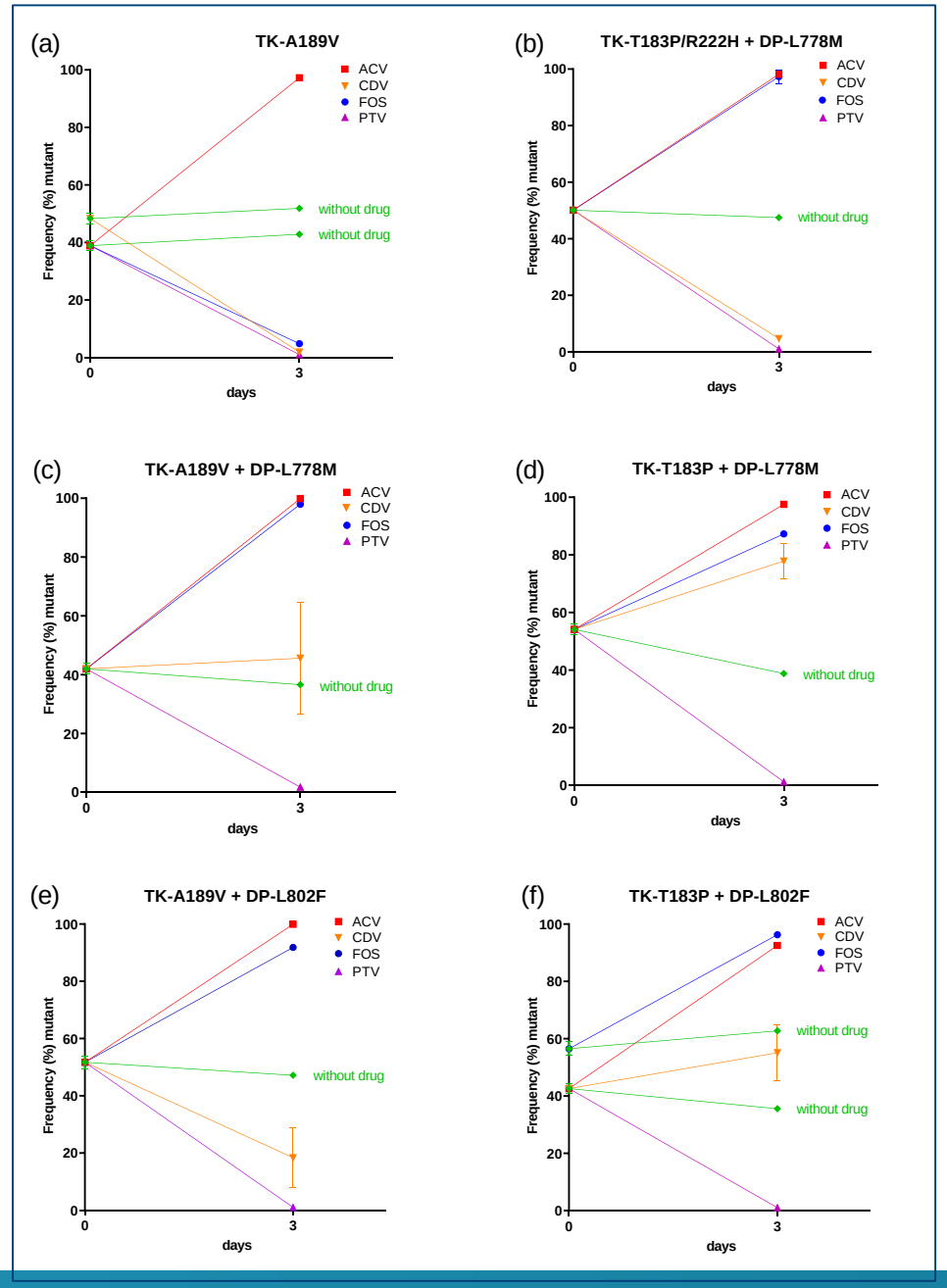

Case study II: a 41-year-old male with GATA-binding factor 2 (GATA2) deficiency / HSCT

- Dual infection competition assays

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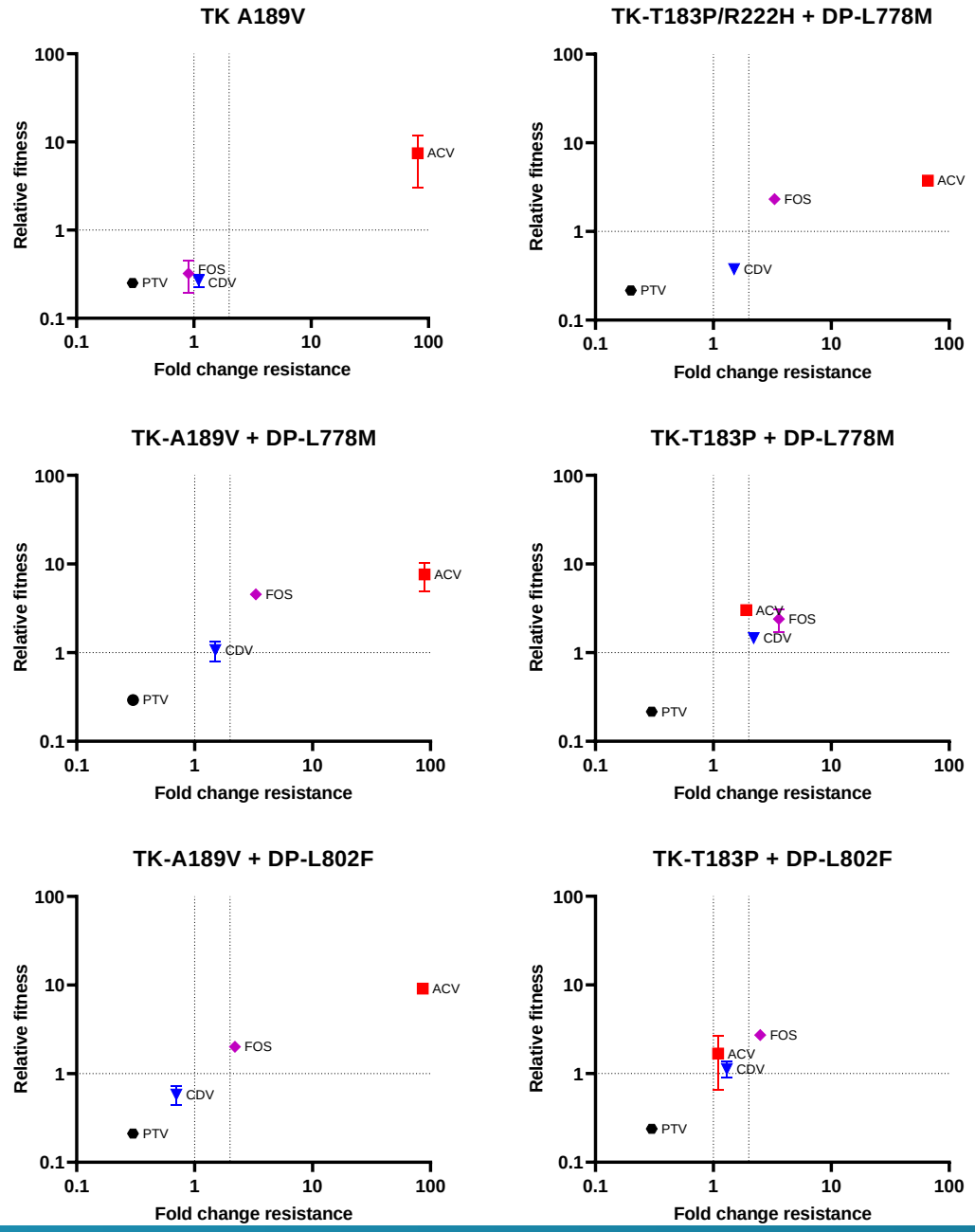

Case study II: a 41-year-old male with GATA-binding factor 2 (GATA2) deficiency / HSCT

- Association between drug-resistance levels and relative viral fitness of HSV-1 mutants.
- Relative fitness (RF), was plotted as a function of the level of drug resistance (mean fold resistance)

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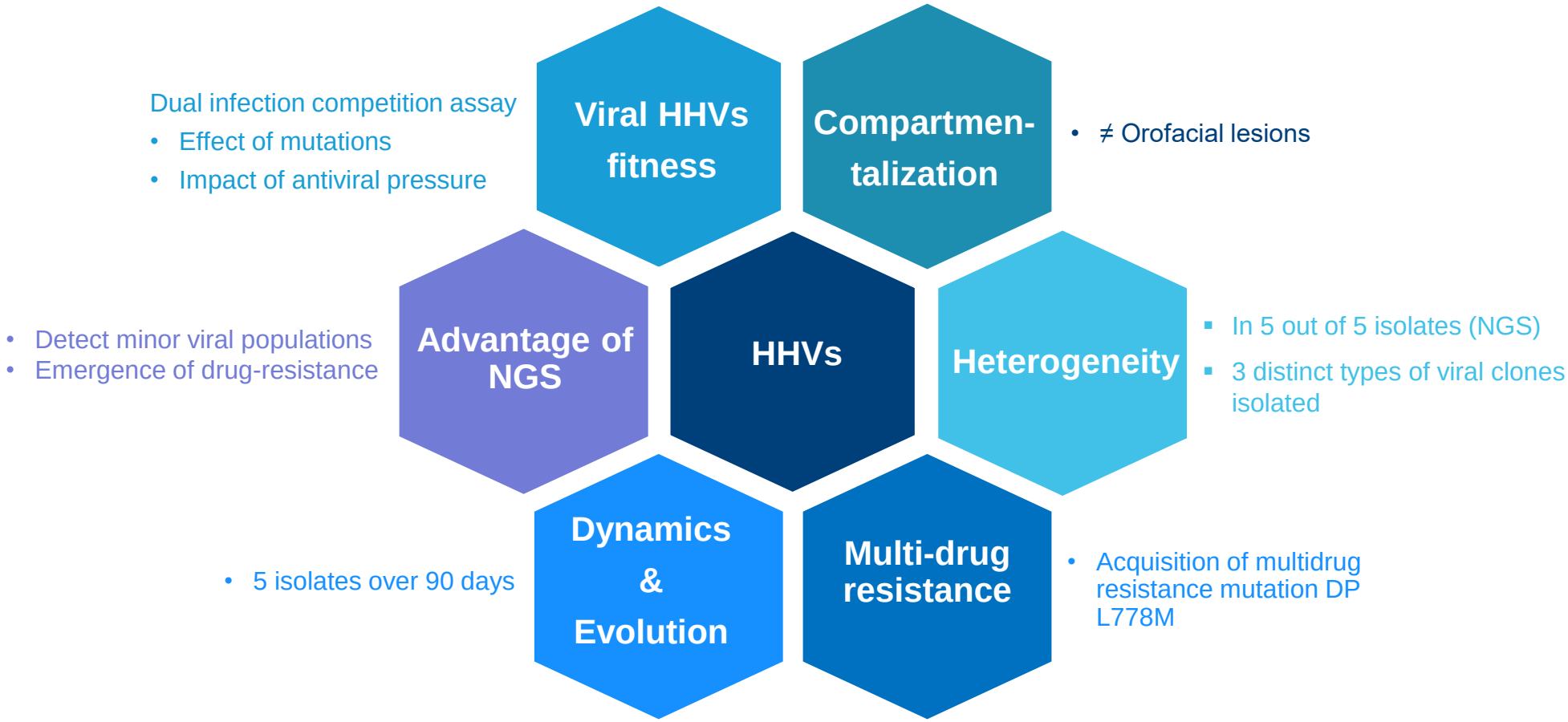
Case study II: a 41-year-old male with GATA-binding factor 2 (GATA2) deficiency / HSCT



Herpesvirus genotyping

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

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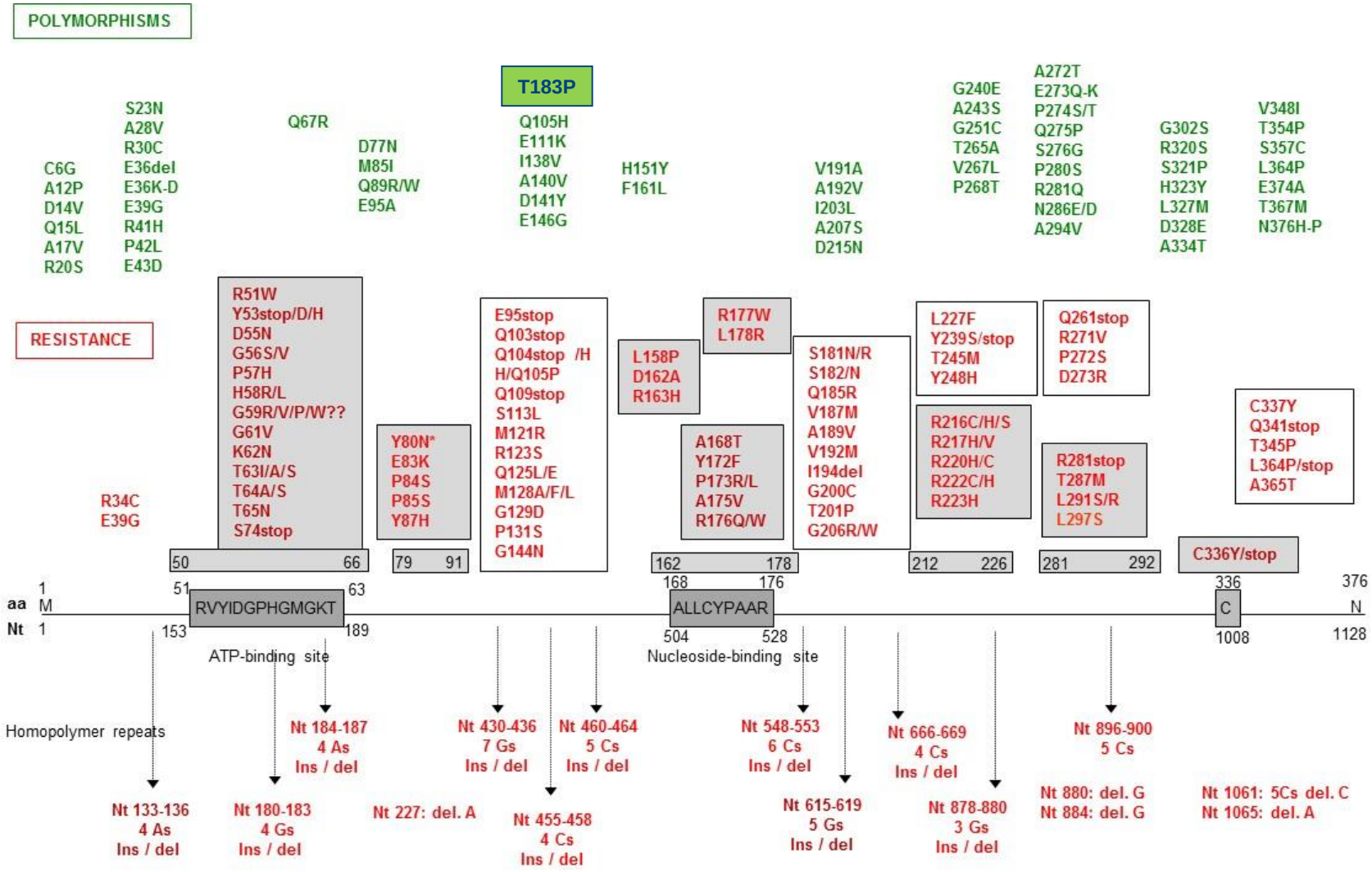
HSV-1 thymidine kinase



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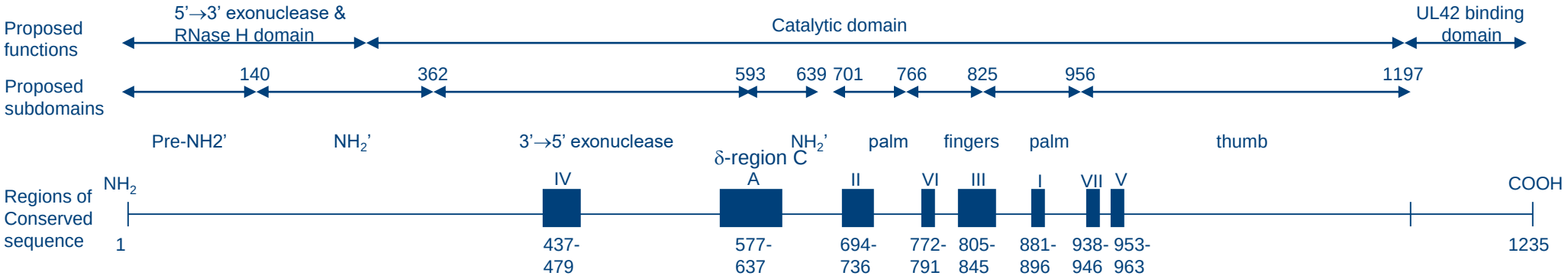
HSV-1 DNA polymerase



Mutations known to confer altered sensitivity to ACV, PFA and/or aphidilcolin. (*hypersensitivity to ACV)

D368A E370A V462A K532T Y557S Q570R L583V Y577H* D581A* E597K/D A605V Y696H R700G V715G/M A719V/T S724N L774F L778M D780N L782I P797T V813M N815S/T N815L/V/Y/E Y818C T821M G841S/C R842S Y941H N961K S889A F891C/Y V892S

Mutations lethal to the virus

E460D G464V K522E P561S Q727R

Novel mutations selected *in vitro* under PFA or PME derivatives and characterized in our Laboratory

Q618H V714M L802F T821M R959H D1070N

Mutations selected *in vitro* under HPMP derivatives and characterized in our Laboratory

V573M R700M K960R W998L L1007M I1028T

Mutations found in clinical isolates associated with drug-resistance found in our previous studies.

H386Y A542S E798K I922T

Genetic polymorphisms

G12E A20V F23I A27T S33G E70D D72N A78V/D A102T K104Q D114A R116H G132C P137Q A138E F171S A229T A232T F248L R330A S338L I352V E421D N425T T434M D418N N522S A566T T639I A646T D660E E684D D672N E675A D703N R700K V743M N711K G749D L753M P875S A899V V904M V905M M906V K908R P920S A995F E1005K I1026S E1082K A1099T D1103H E1104D S1123L-C P1124H A1128V G1129V L1166W E1194D P1199Q A1203T A1204T T1208A A1209T T1219M

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