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RegaVir platform: Case discussions antiviral resistance testing

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Drug-resistance among herpesviruses

- Virtually not observed in immunocompetent individuals
- Well-recognized problem among ≠ populations of immunocompromised patients

Analysis

✓ Emergence of (multi)-drug resistance

✓ Dynamics and compartmentalization

→ evaluation of multiple samples

≠ body sites

≠ time points

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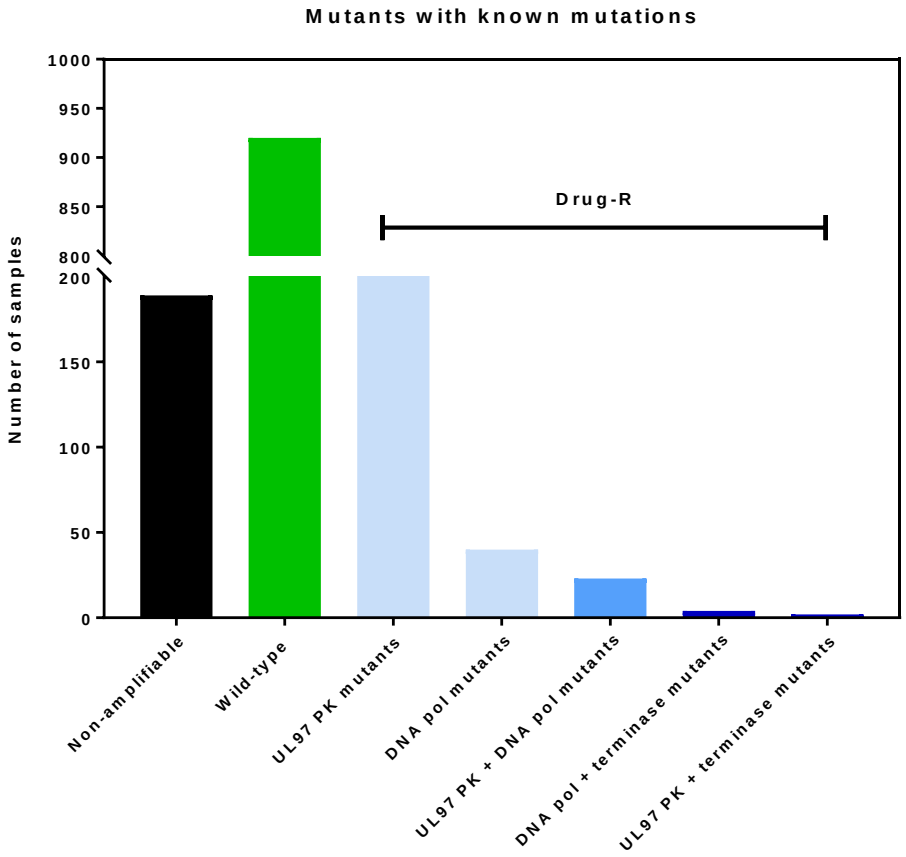
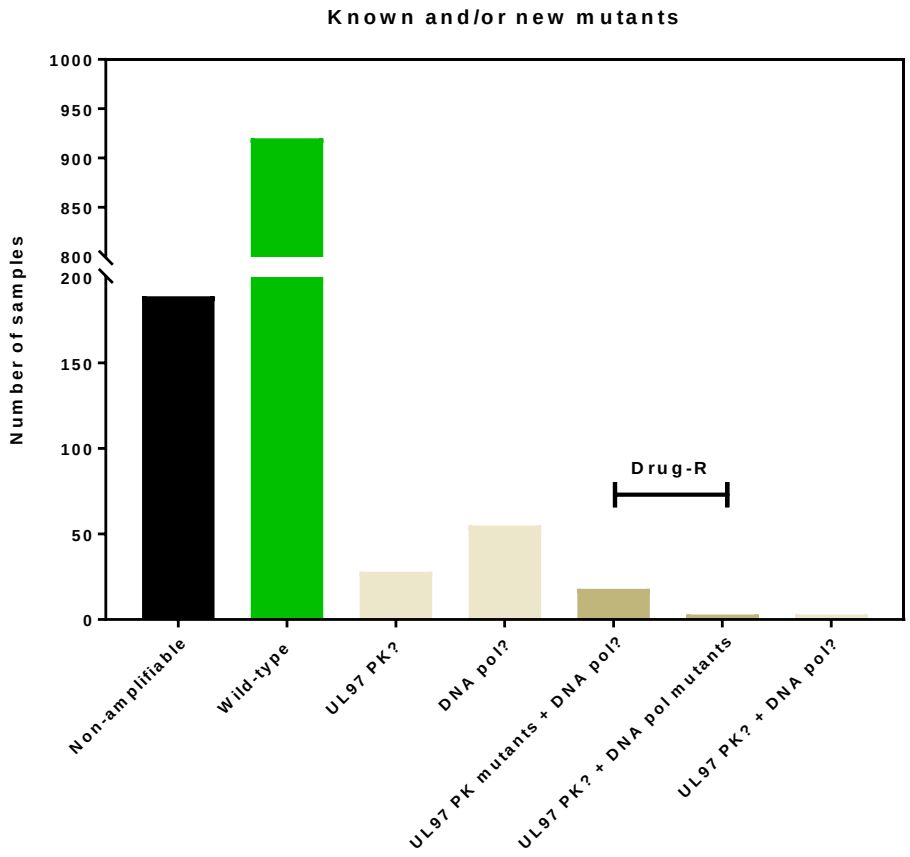
Analysis of HCMV samples from patients that fail antiviral therapy





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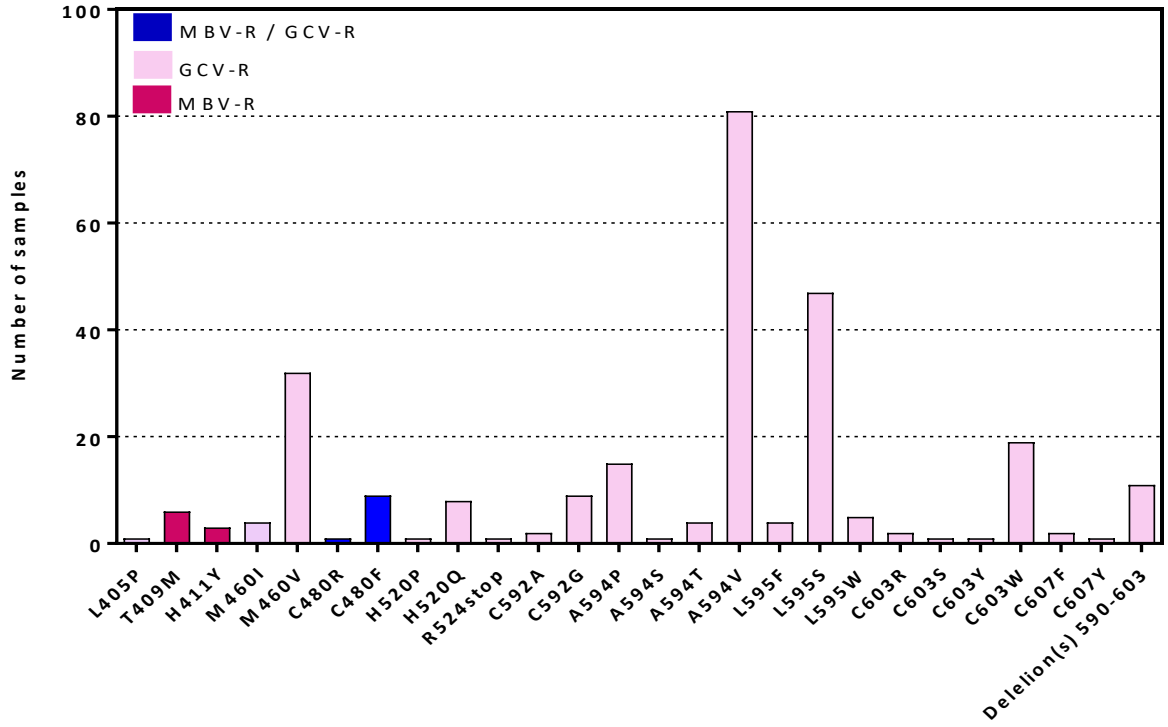
Frequency of drug-resistance mutations identified in the UL97 protein kinase



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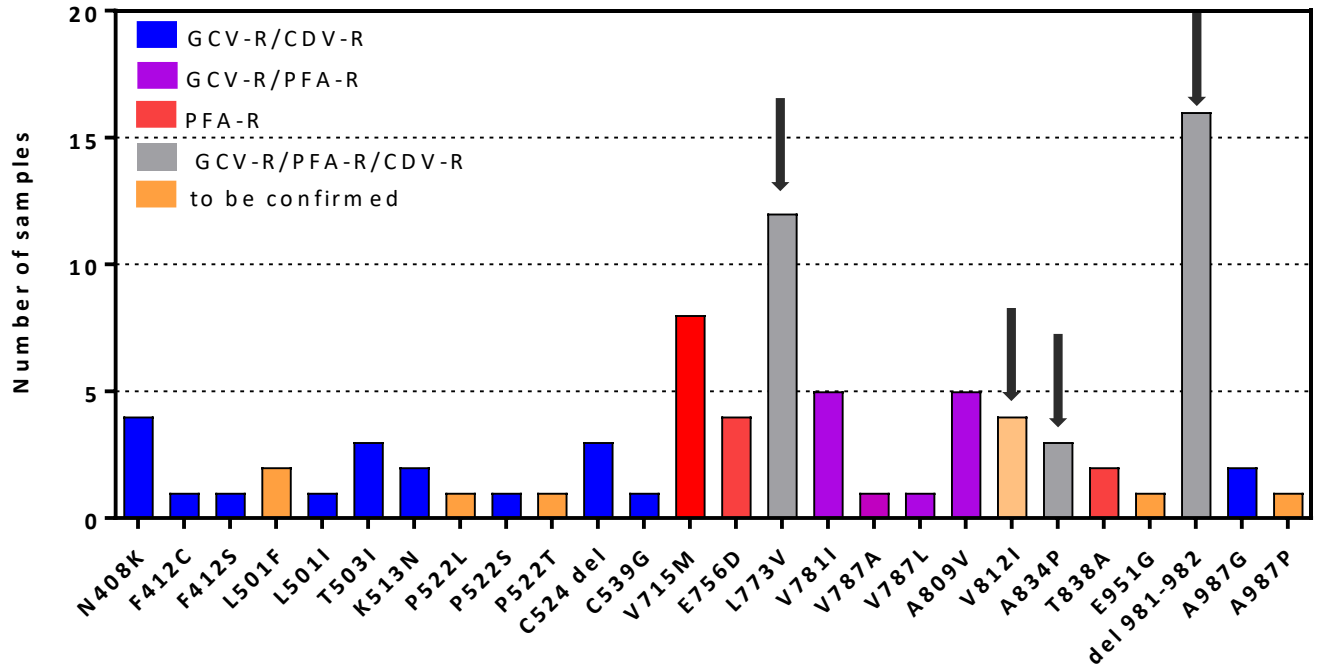
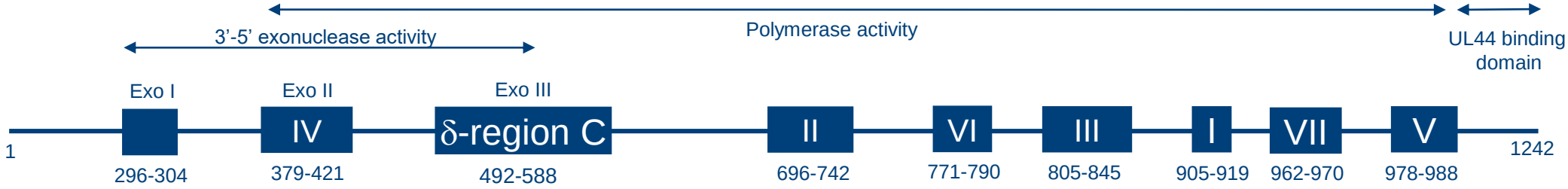
Frequency of drug-resistance mutations identified in the DNA polymerase





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Emergence of HCMV multi-drug-resistance

- **Confered by single mutations associated with resistance to ≠ antivirals**
  - **HCMV DNA pol: del 981-982**
    - ***Pediatric HSCT patient***
    - **Renal transplant recipient (D^+/R^-)**
    - Lung transplant recipient with primary HCMV infection
  - **HCMV DNA pol: A834P**
    - ***Pediatric HSCT patient***
    - Adult HSCT patient
  - **HCMV DNA pol: V812I**
    - HIV seropositive / HSCT patient
  - **HCMV DNA pol: L773V**
    - Adult HSCT patient
    - Pediatric HSCT patient
    - Adult patient with neuroblastome stage IV

- **Confered by co-infection with ≠ strains bearing specific mutations**
  - Lung transplant recipient
  - Kidney transplant recipient undergoing transplectomy

Renal transplant recipient (D^+/R^-)
Multi-drug-resistance CMV infection (DNA pol del. 981-982)

- A 43-year-old female patient (**CMV-seronegative**) with end-stage renal failure due to obstructive renal disease received a kidney transplant from a 36-year-old **CMV-seropositive deceased donor (with CMV IgM and IgG positive).**

- The immunosuppressive regimen consisted of tacrolimus, mycophenolate mofetil, corticosteroids and induction with basiliximab.

- **Valganciclovir for CMV prophylaxis** was initiated on day 5 post-transplantation at **450 mg 1x/day.**

Renal transplant recipient (D^+/R^-)
Multi-drug-resistance CMV infection (DNA pol del. 981-982)

- One week after transplantation, CMV PCR was negative in blood and the patient had excellent kidney function.
- Day 29 post-transplantation: CMV PCR became positive.
- CMV viral load increased, and she developed epigastric pain and diarrhea without fever.
- Day 43 post-transplantation: the dose of valganciclovir was then augmented to 2 × 450 mg/d. Epigastric pain disappeared but diarrhea persisted.

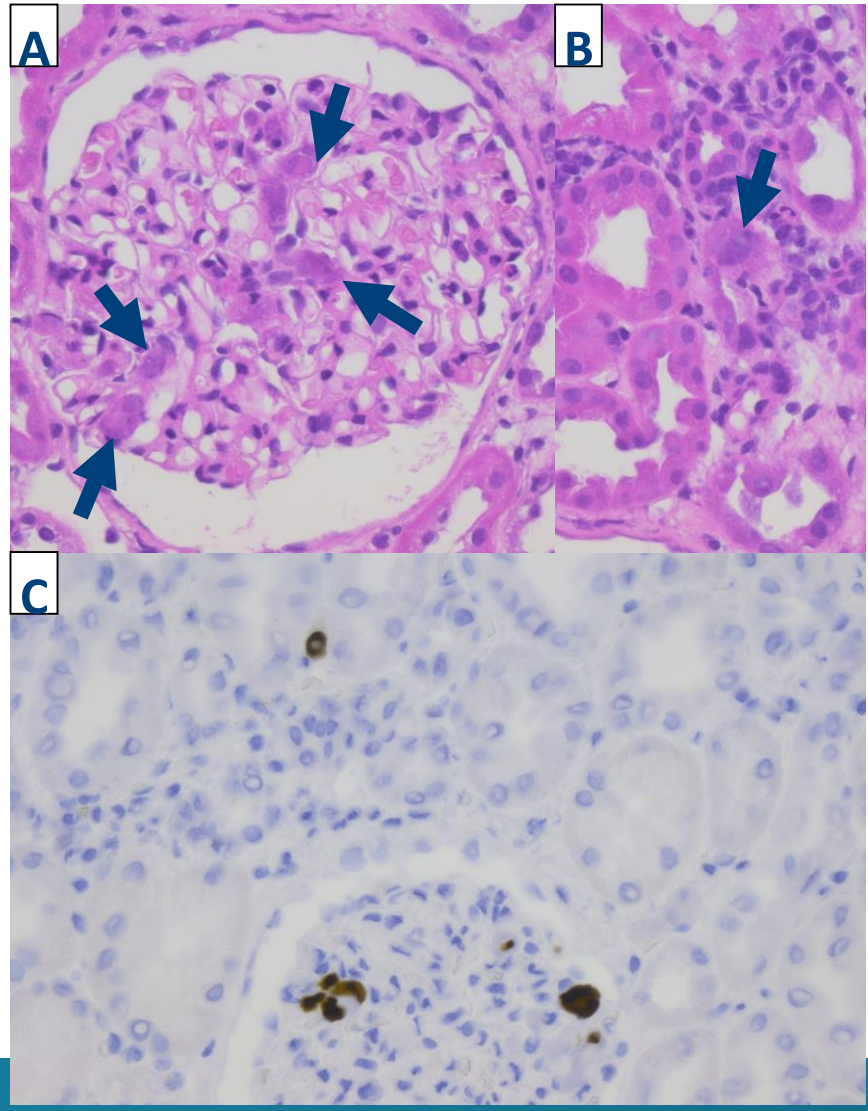
Renal transplant recipient (D^+/R^-)
Multi-drug-resistance CMV infection (DNA pol del. 981-982)

- The viral load rose despite the increased valganciclovir dosing.
- Day 67 post-transplantation, a blood sample (**RV-1050**) was evaluated for drug-resistance, and it can be considered as a **baseline viral genotype** (**wild-type**).
- Day 71 post-transplantation, she was hospitalized for mild liver dysfunction, anorexia, and **acute renal functional deterioration** that partly recovered.
- The **CMV viral load increased** to 6.4 log copies/mL. Because RV-1050 presented a wild type CMV genotype, **valganciclovir was continued at a dosage of 2 × 450 mg/day.**

Renal transplant recipient (D^+/R^-)
Multi-drug-resistance CMV infection (DNA pol del. 981-982)

- Day 109: kidney allograft biopsy for graft dysfunction showed clear **T-cell mediated rejection** with tubulitis grade 2 and interstitial inflammation grade 3.

- **Anti-rejection treatment with high dose corticosteroids was started.**
- The kidney biopsy (CS_3_3) showed renal tubular epithelial cells with nuclear inclusions that stained positive for CMV confirming the diagnosis of CMV nephropathy



The kidney biopsy showed several glomerular (A) and tubular (B) cells with enlarged nuclei, containing inclusions (arrows). These showed immune reactivity for CMV (C), confirming the diagnosis of CMV nephropathy.

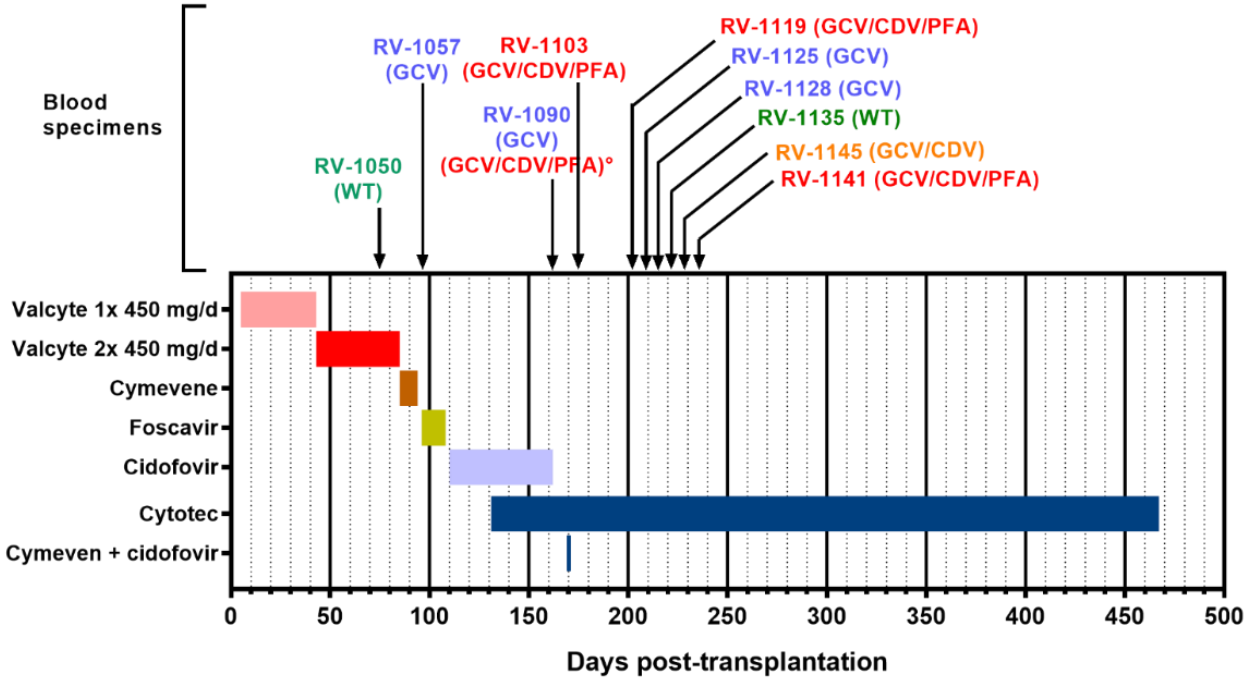
Renal transplant recipient (D^+/R^-)
Multi-drug-resistance CMV infection (DNA pol del. 981-982)

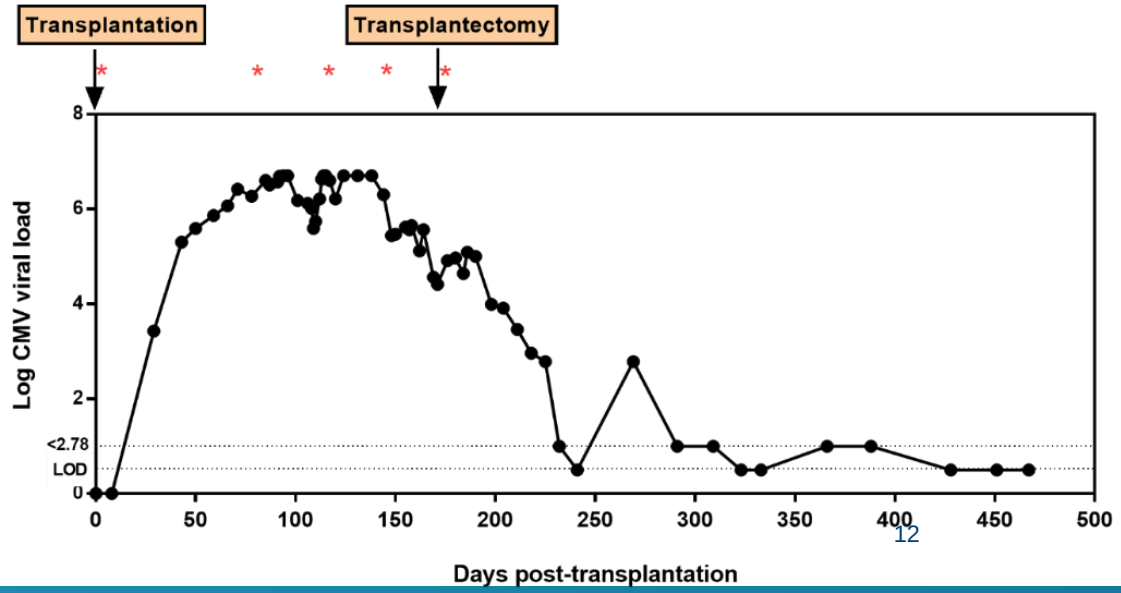
- Day 109: Retrospective genotyping by Sanger sequencing indicated:

  - a mix of wild type and A594V UL97 mutant in the **kidney biopsy** (CS_3_3)

  - heterogeneous populations of UL97 A594V and C592G mutants in the **blood** (CS_3_8)

→ **Compartmentalization**





Overview of CMV-DNA PCR in relation to time after kidney transplantation.
Anti-CMV therapy and samples were genotypically analyzed prospectively (by Sanger sequencing) or retrospectively (by NGS).
Abbreviations: WT: wild-type, GCV: ganciclovir, CDV: cidofovir, PFA: foscarnet.

Between brackets are indicated the drugs against which resistance was determined following conventional CMV genotyping.
°Indicates resistance as determined retrospectively by NGS differing from Sanger sequencing.
*Transplant biopsy performed.
Cytotec: CMV Ig.

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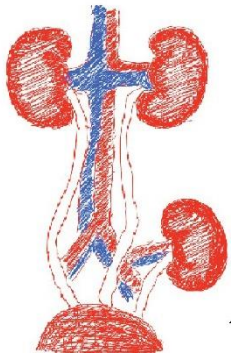
Renal transplant recipient (D^+/R^-)
Multi-drug-resistance CMV infection (DNA pol del. 981-982)

Blood sample	Days post-transplantation	Date	Sanger sequencing
RV-1050	**67**	11/08/2016	wild-type
RV-1057	**88**	01/09/2016	UL54: wild -type UL97: C592G mixed
RV-1090	**152**	04/11/2016	UL54: wild -type UL97: A594V mixed
RV-1103	**165**	17/11/2016	UL54: del 981-982 mixed UL97: A594V mixed
RV-1119	**192**	14/12/2016	UL54: del 981-982 mixed UL97: A594V mixed
RV-1125	**198**	20/12/2016	UL54: wild -type UL97: A594V mixed
RV-1128	**204**	26/12/2016	UL54: wild -type UL97: A594V mixed
RV-1135	**211**	02/01/2017	UL54: wild-type UL97: wild-type
RV-1145	**218**	09/01/2017	UL54: P522T UL97: A594V
RV-1141	**1141**	16/01/2017	UL54: del 981-982 mixed UL97: A594V mixed

Graft loss
↓immunosuppression
Stop antiviral therapy



DNA pol del. 981-982 associated with multi-drug-resistance

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Day PT	Sample ID	CMV DNA (log)	Specimen type	Analysis performed	Amino acid changes related to resistance in:		Amino acid changes related to natural genetic polymorphisms in:		Resistance to:
					UL97 (protein kinase)	UL54 (DNA polymerase)	UL97 (protein kinase)	UL54 (DNA polymerase)	
0	CS_3_1 (B-1825736)	NA***	Transplant kidney biopsy	Retrospective (Sanger)	Not amplifiable		Not amplifiable		/
67	RV-1050	6.07	Blood	Prospective (Sanger)	None	None	None	S897L	Wild-type
				Retrospective (NGS)	None	None	None	S897L	Wild-type
87	CS_3_2 (B-1836685)	NA	Kidney transplant protocol biopsy	Retrospective (Sanger)	None	None	None	S897L	Wild-type
88	RV-1057	6.51	Blood	Prospective (Sanger)	C592G*	None	None	S897L	GCV
				Retrospective (NGS)	C592G (10.97)[a] A594V (10.75)	None	None	S897L (99.60)	GCV
109	CS_3_3 (B-1839887)	NA	Kidney biopsy for graft dysfunction	Retrospective (Sanger)	A594V*	None	None	S897L	GCV
109	CS_3_8	NA	Blood	Retrospective (Sanger)	A594V* C592G*	None	None	S897L	GCV
152	RV-1090	5.47	Blood	Prospective (Sanger)	A594V*	None	None	S897L	GCV
				Retrospective (NGS)	C592G (2.09) A594V (68.44)	T503A (1.59) P522S (1.73) Δ 981-982 (3.03)	None	S897L (99.72)	GCV, CDV, PFA

*Heterogeneous population of mutant and wild-type virus detected by Sanger sequencing.
[a]Percentage of subpopulation variants detected by deep sequencing (Illumina platform).
NA: not available.

Day PT	Sample ID	CMV DNA (log)	Specimen type	Analysis performed	Amino acid changes related to resistance in:		Amino acid changes related to natural genetic polymorphisms in:		Resistance to:
					UL97 (protein kinase)	UL54 (DNA polymerase)	UL97 (protein kinase)	UL54 (DNA polymerase)	
157	CS_3_4 (B-1846802)	NA	Kidney biopsy for graft dysfunction	Retrospective (Sanger)	Not amplifiable		Not amplifiable		/
158	CS_3_9	NA	Blood	Retrospective (Sanger)	A594V*	None	None	S897L	GCV
165	RV-1103	5.56	Blood	Prospective (Sanger)	A594V*	Δ 981-982*	None	S897L	GCV, CDV, PFA
				Retrospective (NGS)	A594V (22.86)	P522S (1.27) Δ 981-982 (37.37)	None	S897L (99.87)	GCV, CDV, PFA
165	CS_3_7 (B-1847724-01-03)	NA	Transplantectomy kidney biopsy	Retrospective (Sanger)	A594V*	T503A*	None	S897L	GCV, CDV
192	RV-1119	5.0	blood	Prospective (Sanger)	A594V*	Δ 981-982*	None	S897L	GCV, CDV, PFA
198	RV-1125	3.99	blood	Prospective (Sanger)	A594V*	None	None	S897L	GCV
204	RV-1128	3.91	blood	Prospective (Sanger)	A594V*	None	None	S897L	GCV
211	RV-1135	3.46	blood	Prospective (Sanger)	None	None	None	S897L	Wild-type
218	RV-1145	2.96	blood	Prospective (Sanger)	A594V	P522S*	None	S897L	GCV, CDV
225	RV-1141	2.78	blood	Prospective (Sanger)	A594V*	Δ 981-982	None	S897L	GCV, CDV, PFA

*Heterogeneous population of mutant and wild-type virus detected by Sanger sequencing.
*Percentage of subpopulation variants detected by deep sequencing (Illumina platform).
NA: not available.

This case report is important in several aspects

- **Importance of drug-resistance monitoring** over time for tailored antiviral therapy.

- The case is marked by the **rapid evolution of drug-resistant CMV** mutants related to persistently high blood viral loads.

- Next to the direct consequences of CMV infection also **indirect consequences** are present in this case report, as the patient developed **acute graft rejection**.

- **Transient and incomplete response to various anti-CMV treatments**.

This case report is important in several aspects

- **Compartmentalization**: dissimilar CMV viral populations in different body compartments.
- **Increased ability of certain viral mutants to replicate in the graft.**
- By using NGS, **the limitations of Sanger sequencing** to detect minor viral subpopulation are put forward.
- **Careful molecular diagnostics** in our patient allowed optimal treatment options and support in the difficult but eventually **live-saving decision to perform transplantectomy.**

Risk of acute allograft rejection

- **CMV increases the risk of acute allograft rejection and interstitial fibrosis** with tubular atrophy in the kidney graft by inducing an **excessive immune reaction** due to upregulation of cell adhesion molecules, increased expression of human leukocyte antigens and activation of cytotoxic T cells (*Opelz and Dohler, 2015; Reischig et al., 2006).*

- The risk of CMV infection is **highly dependent on the donor and recipient serological status.**

- Besides the donor/recipient serological status, **short prophylaxis duration and higher levels of immunosuppressive therapy are risk factors for developing CMV disease** (Hasegawa et al., 2017).

  → **Universal prophylaxis**

Universal prophylaxis

- **Administration of antivirals to all patients at high risk for CMV, i.e. D+, R-** is recommended in the early post-transplantation period up to 6 months to increase graft survival in patients lacking CMV-specific immunity *(Komorowska-Jagielska et al., 2018; Kuo et al., 2010).*

- **Valganciclovir is currently the most commonly used drug for CMV prophylaxis** because of its improved bioavailability relative to ganciclovir *(Kotton et al., 2018).*

- The usual dose of **valganciclovir for prophylaxis is 900 mg daily *versus* treatment dose (900 mg twice daily),** although this needs to be adjusted for the variable kidney function, as was done in the case of our patient.

- **Valganciclovir 450 mg daily is also effective for CMV prophylaxis** and is associated with lower risk for hematological side effects than the high dose *(Gabardi et al., 2015Halim et al., 2016; Heldenbrand et al., 2016; Stevens et al., 2015).*

Universal prophylaxis

- According to a systematic review and meta-analysis, **valganciclovir 900 mg and 450 mg daily dosing are equipotent for CMV prophylaxis in all-risk renal transplant recipients** at least within the first year of transplantation with no differences regarding acute rejection, allograft loss, mortality, opportunistic infections, premature discontinuation of valganciclovir treatment and leukopenia *(Xin et al., 2017).*

- However, **major concerns with the 450 mg daily dose are higher risk of ganciclovir resistance and breakthrough infection among CMV D+/R- kidney transplant recipients** *(Gabardi et al., 2015; Stevens et al., 2015).*

Renal transplantation & risk for graft loss

- Renal transplant recipients with **both early-onset (<3 months) and late-onset (>3 months) CMV DNAemia with ≥2000 copies/ml are at increased risk for graft loss** *(Reischig et al., 2017).*

- In our case, persistent high viral replication in blood occurred despite implementation of a double valganciclovir dose and reduction of immunosuppression. **Failure to achieve significant viral load reduction or persisting symptomatic disease beyond 2 weeks of antiviral therapy should be interpreted as an inadequate response.**

- **As these clinical features by themselves do not imply that viral drug-resistance is present, genotypic assays for viral drug-resistance mutations should be performed.**

- **Among SOT patients, the median duration of valganciclovir therapy prior to drug-resistance emergence is approximately 22 weeks *(Cherrier et al., 2018)* and foscarnet has been used successfully to manage CMV infections due to UL 97-resistant mutations in D+/R- transplant recipients *(Myhre et al., 2011).***

Renal transplantation & risk for graft loss

- **In our patient, foscarnet & cidofovir failure**
  - At day 157–158 post-transplantation: the UL97 mutant virus could be cleared from the allograft (CS_3_4) but not from the blood (CS_3_9) following cidofovir and foscarnet treatment and reduction of immunosuppressive treatment (A594V UL97 mutant was detected in the blood).

- High levels of CMV replication, unresponsive to different anti-CMV agents, CMV Ig and reduced immune suppression finally lead to the **emergence of a multidrug-resistant circulating CMV infection and graft loss (day165 post-transplantation).**

Renal transplantation & risk for graft loss

- Our patient was at risk for development of CMV drug-resistance with:
  - prolonged antiviral drug exposure
  - ongoing active replication
  - lack of prior CMV immunity
  - strong immune suppression
  - Potentially insufficient drug delivery (*Kotton et al., 2018*)

- Further, the **lack of CMV specific immunity**, which is known to play a critical role in the development and severity of CMV disease, could also be responsible for the failure to clear the virus before transplantectomy.

- Analysis of CMV-specific T-cell frequencies and function is being considered as a **potential biomarker** to predict the patient's ability to control CMV disease (*Egli et al., 2012*).

Primary CMV infection in SOT recipients

- In CMV-naive solid organ transplant recipients, primary CMV infection **usually occurs following reactivation of the latent virus carried in the graft.**

- In our patient, considering that, the **donor had CMV IgM and IgG, indicative of a recent primary infection** with a possibly on-going asymptomatic low viremia, **the donor might have transmitted an active CMV infection to our patient.**

- CMV transmission has been reported from a CMV IgM positive donor only a few days after trans-plantation (*Gangopadhyay et al., 2016*), similar to our patient (CMV PCR positive at day 29 post-transplantation).

Compartmentalization

- Our findings clearly indicate a compartmentalized evolution of the viral subpopulations as highlighted in our previous studies (Bache et al., 2014; Bauters et al., 2016)

  → warrants genotyping of tissue-specific specimens together with blood in patients unresponsive to antiviral therapy.

- ≠ viral mutants can be selected at relatively low amounts in blood where high levels of viral replication take place and **some minor viral variants can invade the graft causing disease.**

  → rapid detection by NGS of minor viral variants should be beneficial!

Compartmentalization

- **C592G UL97, P522S DNA pol & Δ981–982 DNA pol mutants: unable to infect the graft**
- **A594V UL97 and DNA polymerase T503A mutants: able to infect the graft.**

- **Due to:**
  - **Difference in tissue-specific virulence of the viral mutants**
  - **Sampling or local drug concentration issue**

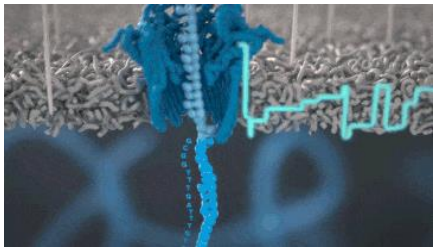
  - Analyzing viral evolution of compartmentalized CMV subpopulations is limited by the **practical difficulty of obtaining tissue-specific samples.**
  - However, **compartmentalized subpopulations should be sought in cases of CMV life-threatening** disease when organ biopsy is performed for graft dysfunction.

Rapid detection of minor viral mutants

- Although NGS provides a more detailed diagnosis of viral mutant subpopulations, the few studies that have analyzed herpesvirus subpopulations by deep sequencing were done retrospectively because specimens are currently processed in batch (Chou, 2015; Chou et al.,2014; Garrigue et al., 2016).

- NGS is becoming more accessible but automation and streamlined processing of samples is still required for *ex tempore* diagnosis of viral drug-resistance to deliver results in a short time.

- Third generation sequencing could be an alternative (such as nanopore sequencing).



Multi-drug-resistance CMV infection & compartmentalization in a **lung transplant with primary CMV infection**

RegaVir code	Date	Type of sample	UL97 genotyping	UL54 genotyping
RV-1091	28/10/2016	blood	**A594V**	Wild-type
RV-1137	05/01/2017	blood	**A594 V**	**Del 981-982***
RV-1179	09/03/2017	blood	**A594 V**	**Del 981-982**
RV-1206	18/04/2017	Eye fluid	**A594 V**	Wild-type

*heterogeneous population

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Multi-drug-resistance CMV infection in a pediatric HSCT recipient

RegaVir code	Date (blood sample)	Virus	Protein kinase genotyping	DNA polymerase genotyping
RV-933	30/12/2015	HHV-6	Wild-type	Wild-type
RV-934	04/01/2016	HHV-6	Wild-type	Wild-type
RV-935	11/01/2016	HHV-6	Wild-type	Wild-type
RV-936	18/01/2016	HHV-6	Wild-type	Wild-type
RV-975	21/03/2016	CMV	Wild-type	Wild-type
RV-1014	08/06/2016	CMV	**A594T*** **L595W***	**A834P**
RV-1028	22/06/2016	CMV	**A594T***	**A834P**

*heterogeneous population

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Lung transplant recipient – CMV reactivation
multi-drug-resistance CMV infection (co-infection with ≠ mutants)

RegaVir code	Date	Type of sample	UL97 genotyping	UL54 genotyping
RV-901	18/11/2015	blood	**L595S**	Wild-type
RV-924	01/01/2016	blood	**L595S***	**K513N (GCV^{R} / CDV^{R})*** **V787L (GCV^{R} / PFA^{R})***

*heterogeneous population

Multi-drug resistance confered by co-infection with ≠ strains bearing specific mutations

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Kidney transplant recipient – CMV reactivation
multi-drug-resistance CMV infection (co-infection with ≠ mutants)

RegaVir code	Date	Type of sample	UL97 genotyping	UL54 genotyping	
RV-1861	23.09.19	Blood	**L595S**	**wt**	
RV-1891	24.10.19	Blood	**L595S**	**wt**	
RV-1904	05.11.19	Stomach biopsy	**L595S**	**wt**	
RV-1931	18.11.19	Kidney Biopsy	**L595S**	**wt**	
RV-1905	05.11.19	BAL	**L595S* + del. KLTHC 599-603***	**wt**	
RV-1996	27.01.20	Blood	**wt**	**N408K* (GCV-R/CDV-R) + E756D* (PFA-R)**	
RV-2062	23.04.20	Blood	**wt**	**N408K (GCV-R/CDV-R) + E756D (PFA-R)**	UL56: C493S?
RV-2074	14.05.20	Blood	**wt**	**N408K (GCV-R/CDV-R) + E756D (PFA-R)**	UL56: C493S?

*heterogeneous population

Multi-drug resistance confered by co-infection with ≠ strains bearing specific mutations

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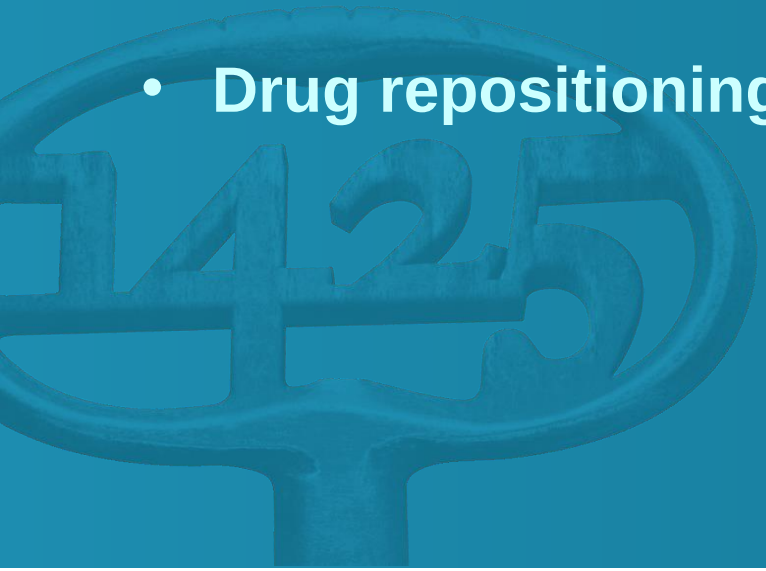
Emergence of multi-drug-resistance

- Clinical challenge to manage multi-drug-resistance
- Urgent need for new antiviral agents
- Investigational drugs for compassionate use?
- Drug repositioning (drug repurposing)?



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