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RegaVir platform: Case discussions antiviral resistance testing

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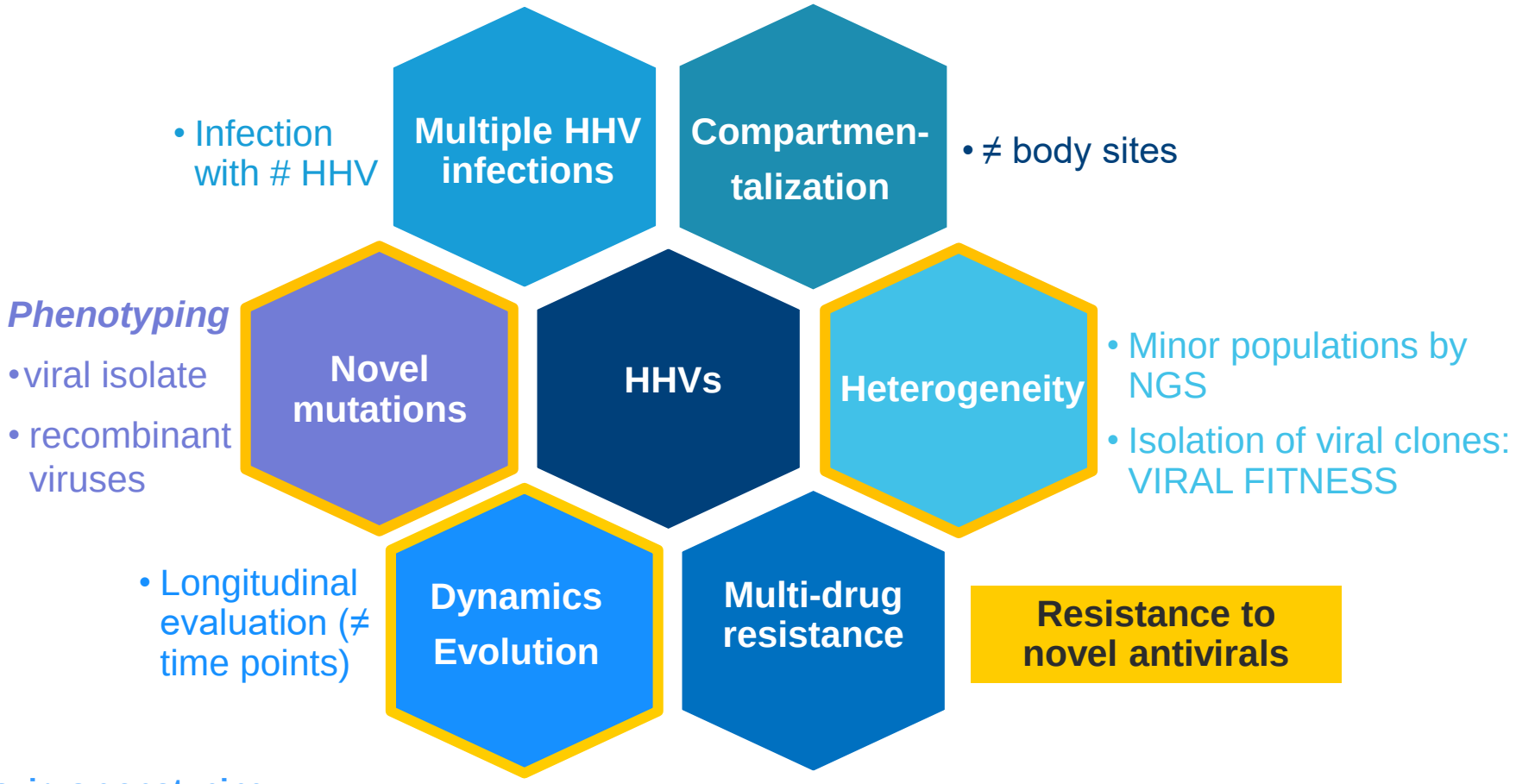
RegaVir platform for translational research



Herpesvirus genotyping

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

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Emergence of HCMV strains with a similar profile in ≠ patients

Patients (UZ Leuven)	RegaVir ID	Date (blood sample)	UL97 genotyping (protein kinase)	UL54 genotyping (DNA pol)
Bowel & pancreas transplantation on 19/03/2017	**RV-1366**	18.12.2017	Wild-type	A505G (GCV-R / CDV-R?)
	RV-1395	02.02.2018	Wild-type	A505G (GCV-R / CDV-R?) **V781I (GCV-R / PFA-R)*** **E951Q (GCV-R / PFA-R)*** **V715M (PFA-R)***
Kidney transplantation on 19/03/2017 (D+/R-) Previous lung transplantation on 21/12/2004 (D-/R-)	**RV-1378**	12.01.2018	Wild-type	A505G (GCV-R / CDV-R?)
Lung transplantation on 19/03/2017	**RV-1389**	17.01.2018	**L595S* (GCV-R)**	A505G* (GCV-R / CDV-R?)
	RV-1445	17.04.2018	**T409M (MBV-R)**	A505G (GCV-R / CDV-R?)
	RV-1473	29.05.2018	**T409M (MBV-R)**	A505G (GCV-R / CDV-R?)

- **Heterogeneity**
- **Novel mutation → recombinant virus**

* Heterogeneous population
A505V: GCV-R / CDV-R

- **Maribavir-R mutations**

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HCMV UL97 protein kinase (pUL97) mutations

Mutations associated with GCV-R
* low levels GCV-R (2-4 fold)
**moderate levels GCV-R (5-15 fold)

Mutations associated with Maribavir-R

Mutations associated with Maribavir-R & ganciclovir-R

L405P*
M460V-I-T**
V466G
C518Y**
H520Q**
P521L
C592G*
C592F**
A594E/T*
A594P-V-G**
L595F-S-W**
E655K**
E596G*
E596Y**
K599T**
C603S*
C603W-R**
C607F*
C607Y**
I610T*
A613V*
Y617del
F342S/Y
V353A
K355del
V356G
K359E/Q
L397R
T409M
H411Y-N-L
F342S
D456N
C480R
Deletions of one or more amino acids in the region 590-603
del590-600**
del591-594**
del595**
del596*
del595-603**
del591-607**
del595-603**
del600*
del601-603**

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HCMV Pol A505V and A505G mutants – recombinant viruses

Strain	Drug	Genotype	Mean EC_{50} (µM)	St Dev EC_{50}	EC_{50} ratio	Published EC_{50} ratios
Control strains						
4198	CDV	Pol WT	0.27	0.06		
4376	CDV	**Pol del 981-982**	0.97	0.20	**3.6**	3.2, 3.7, 3.8
4198	GCV	Pol WT	1.26	0.22		
4376	GCV	**Pol del 981-982**	8.49	2.02	**6.7**	6.2, 7, 7.3
4198	PFA	Pol WT	38.33	6.22		
4376	PFA	**Pol del 981-982**	106.28	22.05	**2.8**	2.7, 3.1, 3.1
Newly constructed mutant strains						
4511	**CDV**	**Pol A505V**	0.67	0.1	**2.5**	2
4513	CDV	Pol A505G	0.37	0.07	1.4	
4511	**GCV**	**Pol A505V**	2.44	0.42	**1.9**	1.8
4513	GCV	Pol A505G	1.67	0.22	1.3	
4511	PFA	Pol A505V	35.77	6.05	0.9	1.1
4513	PFA	Pol A505G	45	6.51	1.2	

Data provided by Sunwen Chou

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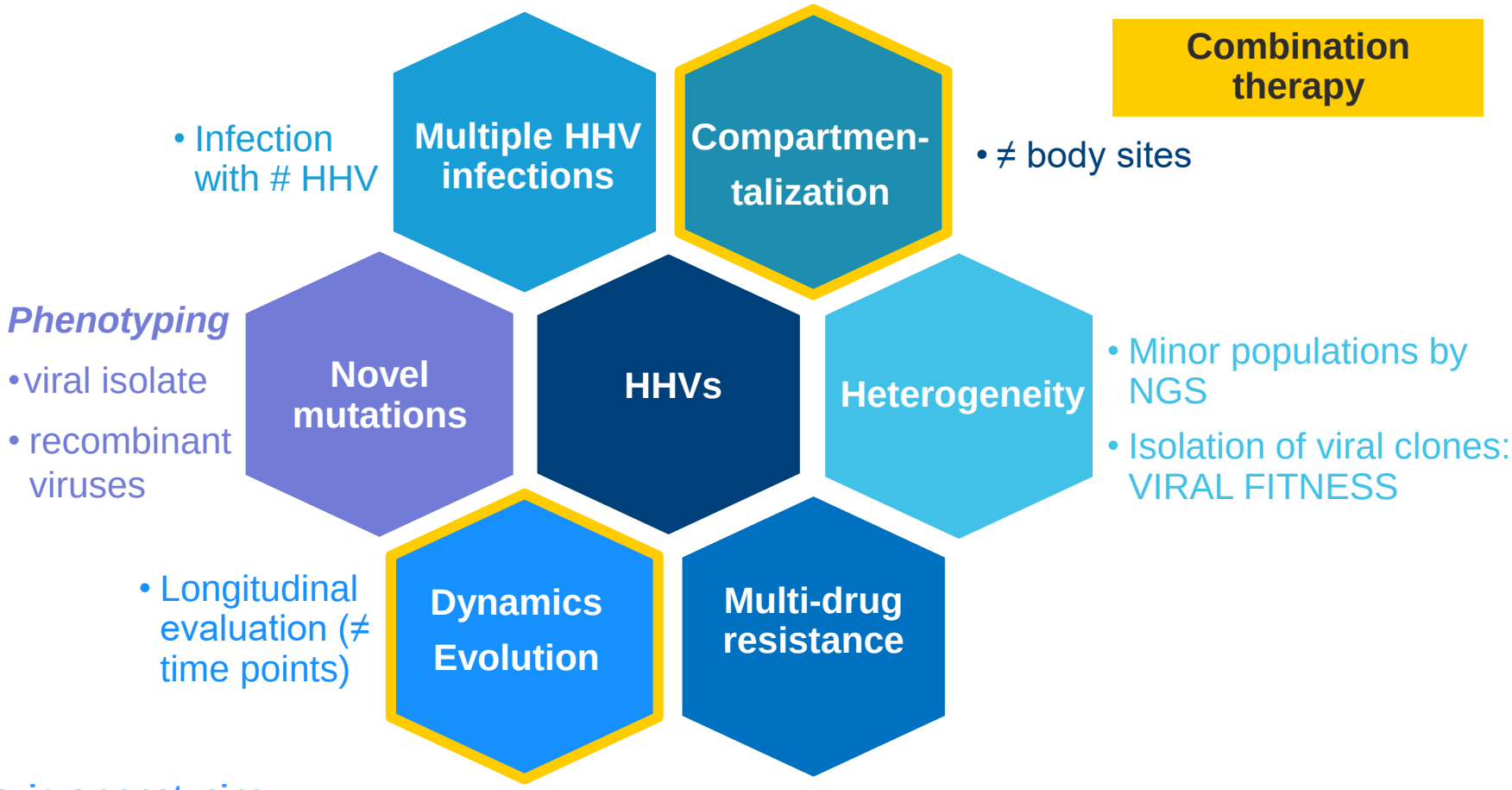
Conclusions

- Importance of **test request form.**
- **Temporal drug-resistance typing to adjust antiviral treatment** is required to adapt antiviral treatment.
- **Emergence of resistance to novel antivirals** (Maribavir) is a concern in the clinic.
- **Viral heterogenicity** is a common phenomenon in HHVs.
- Dangerous to extrapolate the effect of mutations – necessary to **validate the impact of novel mutations on drug-susceptibility.**

RegaVir platform for translational research



Herpesvirus genotyping

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

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Case study

- **A 1280-g girl was born at ~30 weeks' gestation** to an 18-year-old woman.
- **The mother had no history of genital herpes infections** or treatment with ACV and was seronegative for HIV.
- **The infant had a mild respiratory distress syndrome** and was on nasal continuous positive airway pressure since birth.
- Initial antibiotic therapy (ampicillin and gentamicin) was stopped after 5 days, since **bacterial cultures were sterile**.

Case study

- **Day 6**

  Infant developed **severe apnea and bradycardia** and required **oxygen therapy**.

  ✓ **Invasive mechanical ventilation** was started, and chest radiography showed **right upper lung lobe pneumonia.**

  ✓ Therapy was initiated with **vancomycin and ceftazidime. Bacterial cultures were negative.**

- **Day 7**

  ✓ **Vesicular lesions** developed on the trunk and face.

  ✓ Cultures from vesicles and endotracheal tube grew **HSV-2**.

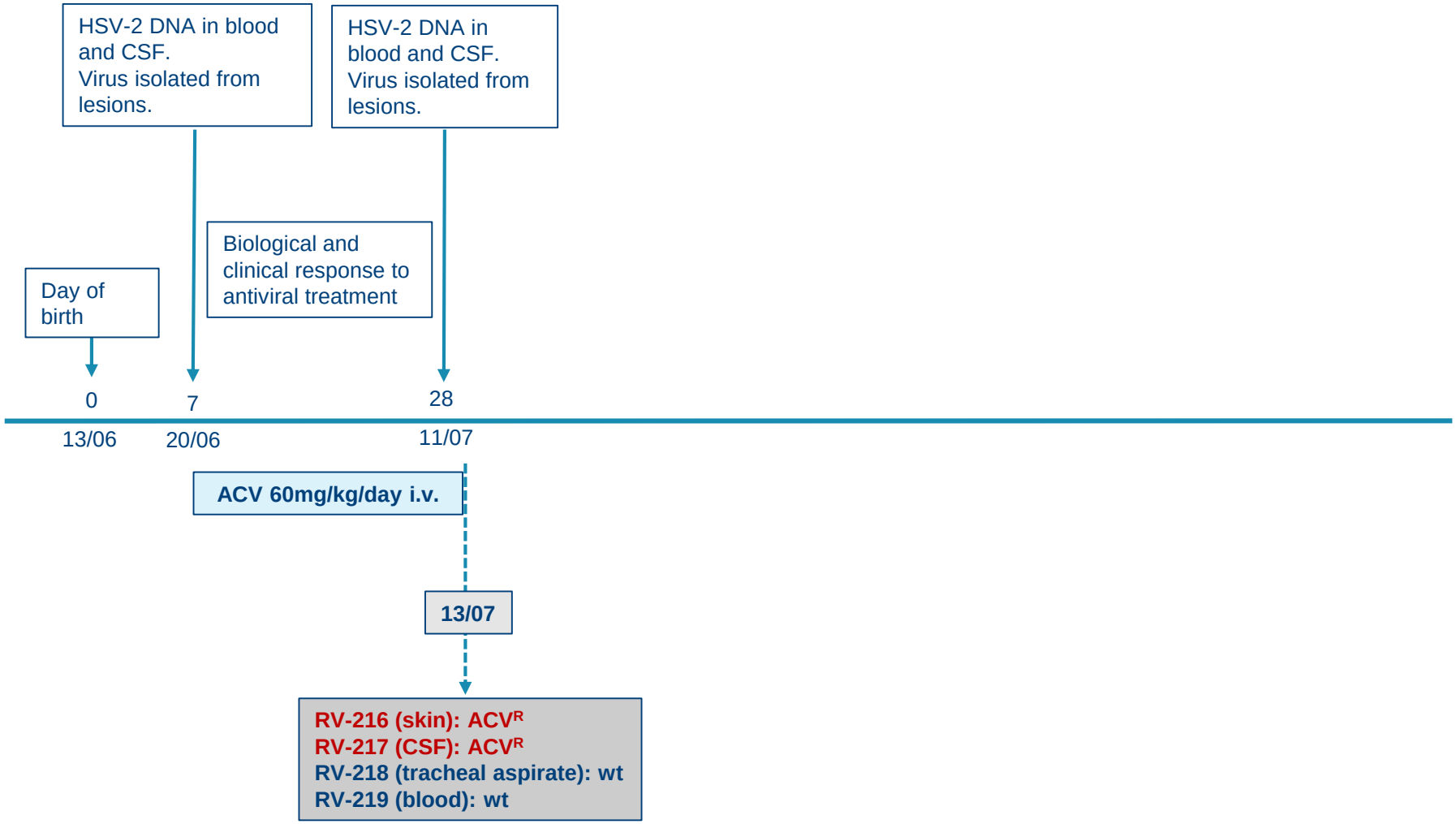
  ✓ **Intravenous ACV therapy (60 mg/kg/day)** was initiated and was continued **for 21 days**, with dosing being adjusted weekly for weight gain throughout the entire administration period.

Case study

- **Day 11**
  - ✓ **HSV-2 Viral DNA** was detected in **blood and CSF** by PCR.
  - ✓ **CSF parameters** showed **elevated protein concentration** (2.17 g/L), **normal glucose concentration**, and **high-count white blood cells** (155/µL with 88% **lymphocytes** for <1 red blood cell/µL).
  - ✓ **Liver function tests were abnormal** with alanine aminotransferase of 58 UI/L, γ-glutamyl transpeptidase of 112 UI/L, and lactate dehydrogenase of 923 UI/L.

Case study

- **Clinical and biological evolution was satisfying** until day 28.
- The neonate developed **new skin lesions** following 21 days of ACV therapy
- **CSF remained positive for HSV-2**
- **Acyclovir therapy** was **continued without interruption** after the completion of the initial 21-day course of therapy
- **Day 30:** different samples were sent to RegaVir for **drug resistance analysis**.
  - ✓ **RV-216 (skin)**
  - ✓ **RV-217 (CSF)**
  - ✓ **RV-218 (tracheal aspirate)**
  - ✓ **RV-219 (blood)**


HSV-2 DNA in blood and CSF.
Virus isolated from lesions.
HSV-2 DNA in blood and CSF.
Virus isolated from lesions.
Biological and clinical response to antiviral treatment
Day of birth
0
7
28
13/06
20/06
11/07
ACV 60mg/kg/day i.v.
13/07
RV-216 (skin): ACV^R
RV-217 (CSF): ACV^R
RV-218 (tracheal aspirate): wt
RV-219 (blood): wt

	Date collected			
	13/07 (day 30)			
Isolate	**RV-216**	**RV-217**	**RV-218**	**RV-219**
Type of sample	Skin	CSF	Tracheal aspirate	Blood
PCR for TK and DNA pol genes	+	+	+	+
Phenotyping	**ACV-R**	Virus not isolated	Virus not isolated	Virus not isolated
TK mutations linked to drug-resistance	**C deletion Nts 551-556**	**C deletion Nts 551-556**	None	None
TK mutations linked to genetic polymorphisms	None	None	None	None
DNA polymerase mutations linked to drug-resistance	None	None	None	None
DNA polymerase mutations linked to genetic polymorphisms	A9T E139K E678G P801T G904A E905A A906G	N.A.	A9T E139K E678G P801T 904A E905A A906G	E678G P801T G904A E905A A906G

Strain	EC_{50} (µg/ml)					
	Acyclovir	Ganciclovir	Brivudin	Foscavir	Cidofovir	Adefovir
RV-216	**13.4** **31.7**	**20** **4**	20 20	29.3 31.9	0.8 0.8	7.2 4.4
Strain G (reference HSV-2 strain)	0.048	0.0064	20	32.7	0.4	4.2

EC_{50} : Concentration required to reduce virus induced cytopathicity by 50%

N.A.: not available due to insufficient material

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Case study

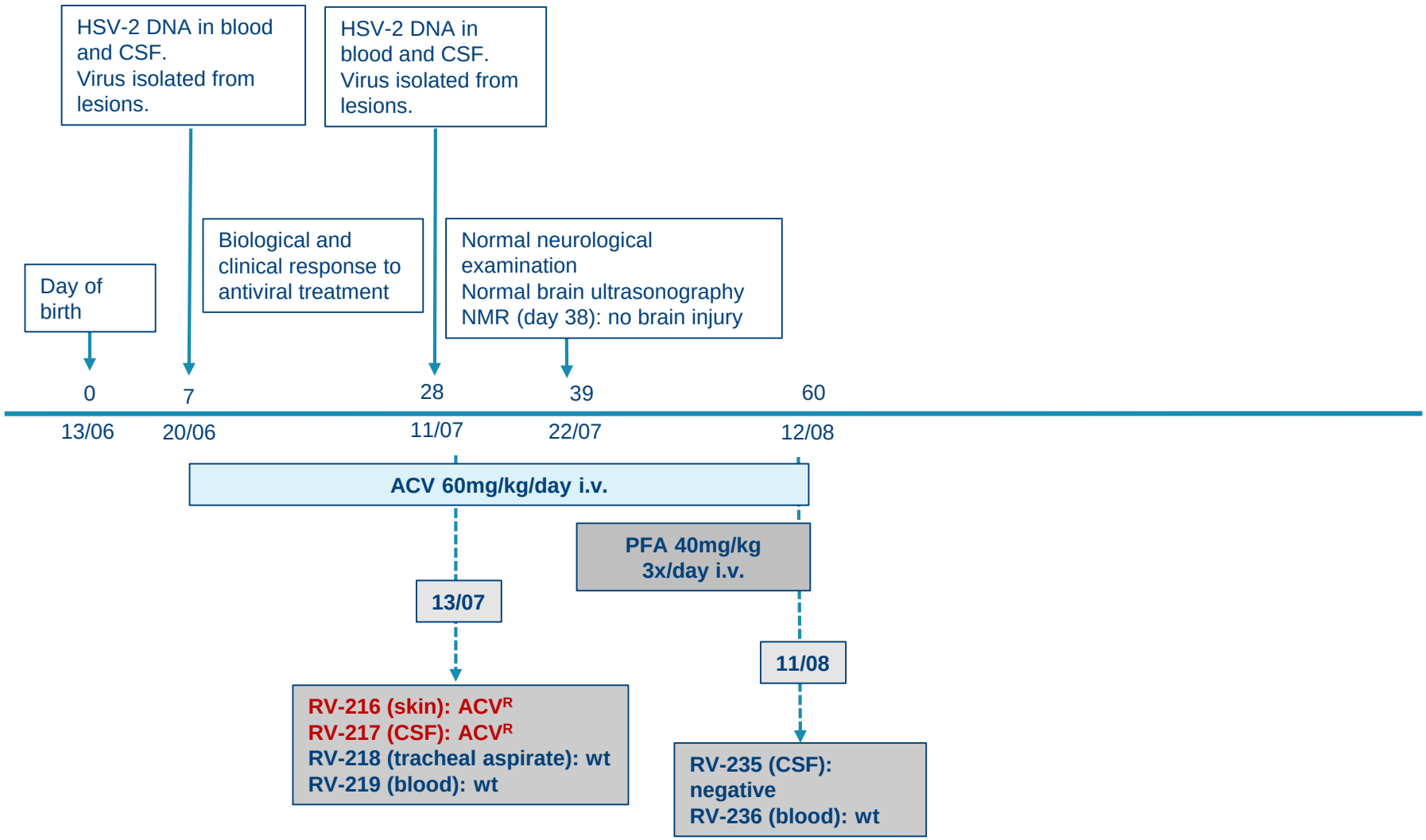
- The infant showed **normal neurological examination** and **brain ultrasonography remained normal**.

- **Day 38:** a nuclear magnetic resonance scan showed **no brain injury caused by HSV-2 infection**.

- **Day 39:** Considering the **virus compartmentalization** in this baby (ACV-R HSV-2 in skin lesions and CSF and wt virus in tracheal aspirate and blood), **PFA (intravenous, 40 mg/ kg/day) was added to the ongoing ACV therapy (60 mg/kg/day)** resulting in improvement of the lesions, and **clearance of the virus in the CSF (RV-235)**.

- No side effects were recorded.

- **After 21 days of combined therapy, HSV-2 in blood could not be detected** by real-time PCR in routine laboratory tests, and **treatment was halted at day 60.**
- However, retrospectively, the TK gene could be amplified the **blood (RV-236)** and revealed a wild-type genotype.


HSV-2 DNA in blood and CSF.
Virus isolated from lesions.
HSV-2 DNA in blood and CSF.
Virus isolated from lesions.
Biological and clinical response to antiviral treatment
Normal neurological examination
Normal brain ultrasonography
NMR (day 38): no brain injury
Day of birth
0
7
28
39
60
13/06
20/06
11/07
22/07
12/08
ACV 60mg/kg/day i.v.
PFA 40mg/kg 3x/day i.v.
13/07
11/08
RV-216 (skin): ACVR
RV-217 (CSF): ACVR
RV-218 (tracheal aspirate): wt
RV-219 (blood): wt
RV-235 (CSF): negative
RV-236 (blood): wt

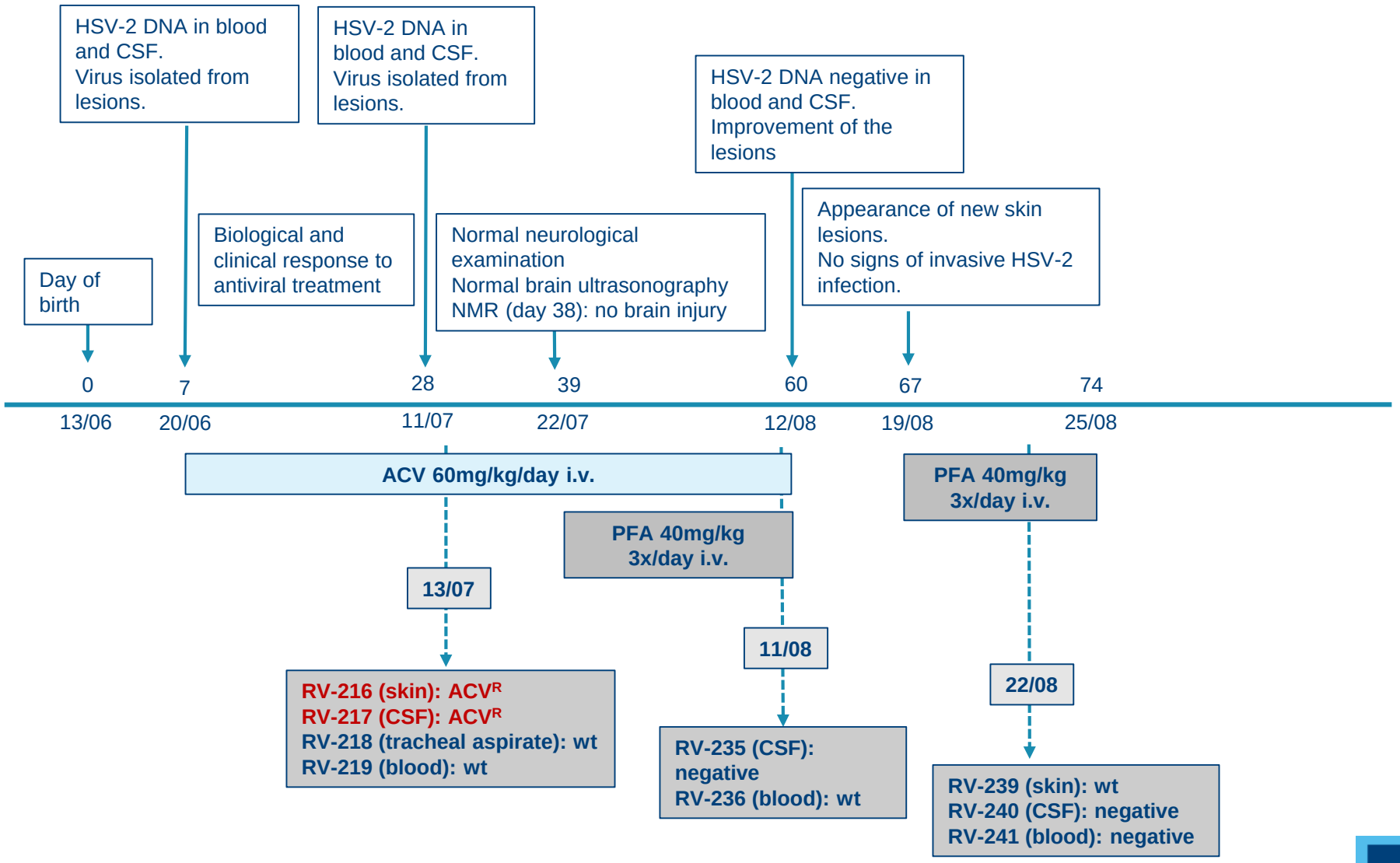
	Date collected								
	13/07 (day 30)				11/08 (day 59)				
Isolate	**RV-216**	**RV-217**	**RV-218**	**RV-219**	**RV-235**	**RV-236**			
Type of sample	Skin	CSF	Tracheal aspirate	Blood	CSF	Blood			
PCR for TK and DNA pol genes	+	+	+	+	-	+			
Phenotyping	**ACV-R**	Virus not isolated	Virus not isolated	Virus not isolated	-	Virus not isolated			
TK mutations linked to drug-resistance	**C deletion Nts 551-556**	**C deletion Nts 551-556**	None	None	-	None			
TK mutations linked to genetic polymorphisms	None	None	None	None	-	None			
DNA polymerase mutations linked to drug-resistance	None	None	None	None	-	N.A.			
DNA polymerase mutations linked to genetic polymorphisms	A9T E139K E678G P801T G904A E905A A906G	N.A.	A9T E139K E678G P801T 904A E905A A906G	E678G P801T G904A E905A A906G	-	N.A.			

N.A.: not available due to insufficient material

Case study

- The infant developed **new skin vesicles** 7 days after stopping antiviral therapy. There were no signs of an invasive HSV-2 infection and PCR in CSF was negative.

- **A second course of PFA therapy** was initiated for 7 days and after 3 days of monotherapy with PFA:
  - ✓ **HSV-2 was undetectable** in **CSF (RV-240)** and **blood (RV-241**
  - ✓ Although HSV-2 could be isolated from the **skin lesions (RV-239)** showing a **wt phenotype and genotype.**


HSV-2 DNA in blood and CSF.
Virus isolated from lesions.
HSV-2 DNA in blood and CSF.
Virus isolated from lesions.
HSV-2 DNA negative in blood and CSF.
Improvement of the lesions
Day of birth
Biological and clinical response to antiviral treatment
Normal neurological examination
Normal brain ultrasonography
NMR (day 38): no brain injury
Appearance of new skin lesions.
No signs of invasive HSV-2 infection.
0
7
28
39
60
67
74
13/06
20/06
11/07
22/07
12/08
19/08
25/08
ACV 60mg/kg/day i.v.
PFA 40mg/kg 3x/day i.v.
PFA 40mg/kg 3x/day i.v.
13/07
11/08
22/08
RV-216 (skin): ACVR
RV-217 (CSF): ACVR
RV-218 (tracheal aspirate): wt
RV-219 (blood): wt
RV-235 (CSF): negative
RV-236 (blood): wt
RV-239 (skin): wt
RV-240 (CSF): negative
RV-241 (blood): negative

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	Date collected								
	13/07 (day 30)				11/08 (day 59)		22/08 (day 71)		
Isolate	**RV-216**	**RV-217**	**RV-218**	**RV-219**	**RV-235**	**RV-236**	**RV-239**	**RV-240**	**RV-241**
Type of sample	Skin	CSF	Tracheal aspirate	Blood	CSF	Blood	Skin	CSF	Blood
PCR for TK and DNA pol genes	+	+	+	+	-	+	+	-	-
Phenotyping	**ACV-R**	Virus not isolated	Virus not isolated	Virus not isolated	-	Virus not isolated	Wild-type	-	-
TK mutations linked to drug-resistance	**C deletion Nts 551-556**	**C deletion Nts 551-556**	None	None	-	None	None	-	-
TK mutations linked to genetic polymorphisms	None	None	None	None	-	None	None	-	-
DNA polymerase mutations linked to drug-resistance	None	None	None	None	-	N.A.	N.A.	-	-
DNA polymerase mutations linked to genetic polymorphisms	A9T E139K E678G P801T G904A E905A A906G	N.A.	A9T E139K E678G P801T 904A E905A A906G	E678G P801T G904A E905A A906G	-	N.A.	E678G P801T G904A E905A A906G	-	-

N.A.: not available due to insufficient material

Strain	EC_{50} (µg/ml)				
	Acyclovir	Ganciclovir	Brivudin	Foscavir	Cidofovir
RV-239	0.064 0.047 0.049	0.0128 0.0128 0.017	8 17.9 40	25.3 32.7 35.8	0.42 0.47 0.47
Strain G (reference HSV-2 strain)	0.029	0.0128	13.7	16	0.30

EC_{50} : Concentration required to reduce virus induced cytopathicity by 50%

	Date collected		
	22/08 (day 71)		
Isolate	**RV-239**	**RV-240**	**RV-241**
Type of sample	Skin	CSF	Blood
PCR for TK and DNA pol genes	+	-	-
Phenotyping	Wild-type	-	-
TK mutations linked to drug-resistance	None	-	-
TK mutations linked to genetic polymorphisms	None	-	-
DNA polymerase mutations linked to drug-resistance	N.A.	-	-
DNA polymerase mutations linked to genetic polymorphisms	E678G P801T G904A E905A A906G	-	-

N.A.: not available due to insufficient material

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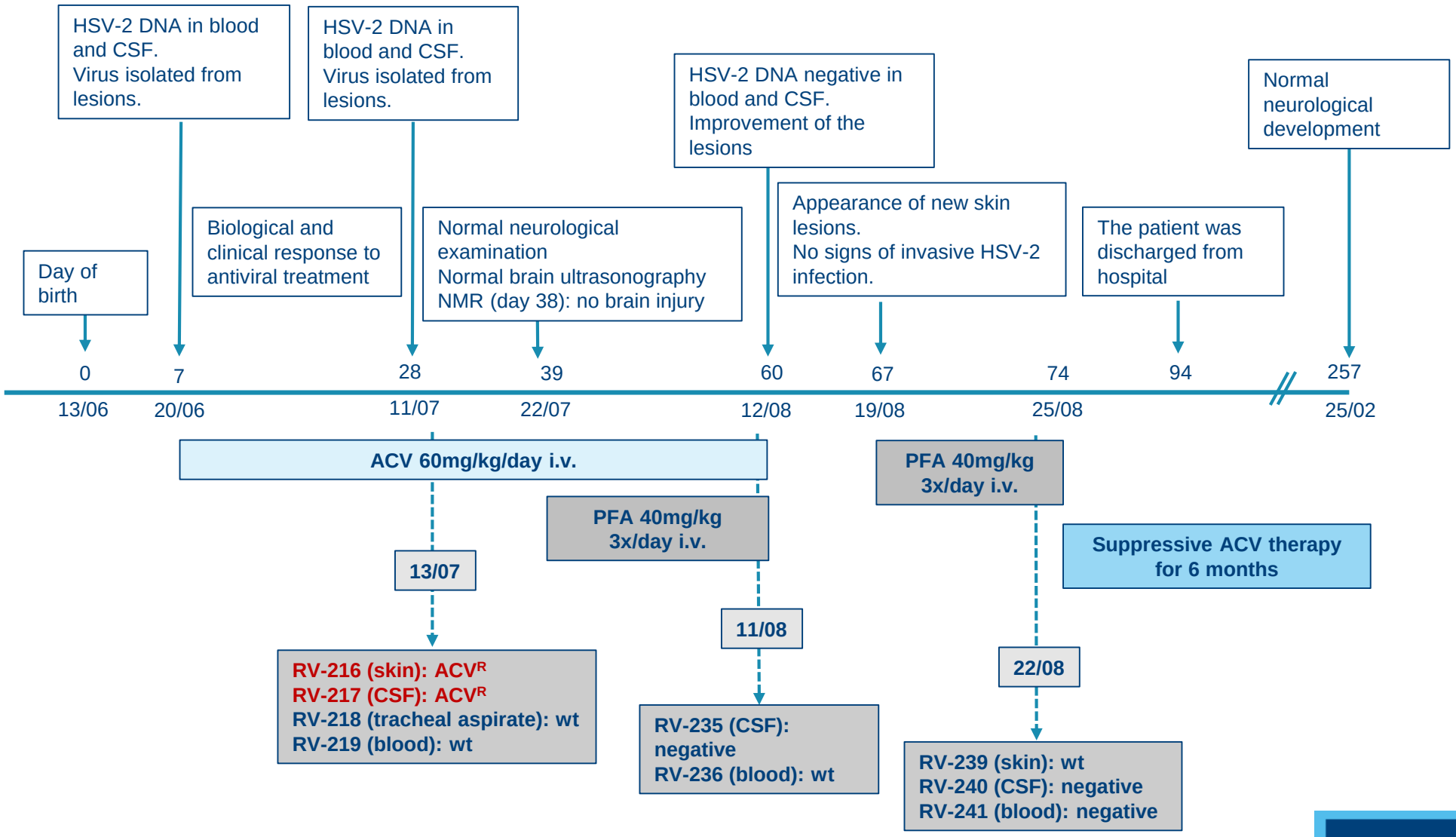
Case study

- **No signs of immunosuppression** were found (normal lymphocyte typing and seronegativity for HIV).
- A **6-month suppressive ACV therapy (300 mg/m^2 per dose orally 3x/day)** on chickenpox doses was initiated.
- The infant **left the hospital on day 94**.
- During the **follow-up**, she presented **repeated herpetic skin lesions** without any signs of generalized infection.
- Her **neurological development** remained completely **normal at 18 months**.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Virus isolated from lesions.
HSV-2 DNA in blood and CSF.
Virus isolated from lesions.
HSV-2 DNA negative in blood and CSF.
Improvement of the lesions
Normal neurological development
Day of birth
Biological and clinical response to antiviral treatment
Normal neurological examination
Normal brain ultrasonography
NMR (day 38): no brain injury
Appearance of new skin lesions.
No signs of invasive HSV-2 infection.
The patient was discharged from hospital
0
7
28
39
60
67
74
94
257
13/06
20/06
11/07
22/07
12/08
19/08
25/08
25/02
ACV 60mg/kg/day i.v.
PFA 40mg/kg 3x/day i.v.
PFA 40mg/kg 3x/day i.v.
Suppressive ACV therapy for 6 months
13/07
11/08
22/08
RV-216 (skin): ACVR
RV-217 (CSF): ACVR
RV-218 (tracheal aspirate): wt
RV-219 (blood): wt
RV-235 (CSF): negative
RV-236 (blood): wt
RV-239 (skin): wt
RV-240 (CSF): negative
RV-241 (blood): negative

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Neonatal HSV infection

- **Relatively rare**, occurring in approximately 1 in 2000–5000 deliveries.
- May be acquired by **intrauterine** (5%), **perinatal** (85%), and **postpartum** (10%) infection.

- **HSV-2 causes ~ 75% of the cases**, HSV-1 accounting for the remainder
- **Classification**
  - ✓ **localized SEM** (skin, eyes, and mouth)
  - ✓ **CNS disease**
  - ✓ **disseminated** (with or without CNS involvement) disease.

- Approximately 50% of all babies with neonatal HSV will have CNS involvement affecting any (often multiple) parts of the brain.

Neonatal HSV infection

	Proportion of cases	Clinical manifestations
SEM	45%	Characteristic vesicular lesions Conjunctivitis, excessive tearing Ulcerative lesions of the mouth, palate, and tongue
CNS disease	30%	Seizures Lethargy Irritability Tremors Poor feeding Skin lesions present in 60 to 70%
Disseminated disease	25%	Sepsis syndrome Fever or hypothermia Hepatitis Respiratory distress Disseminated intravascular coagulopathy Skin lesions present in 60 to 80% CNS involvement in 60 to 75%

Neonatal HSV infection

- The current recommended antiviral regimen is **intravenous acyclovir** (60 mg/ kg/day divided into three doses).
- **Localized SEM has no mortality after antiviral treatment ≠ disseminated and CNS disease.**

- Despite high-dose ACV therapy, **the morbidity in survivors with disseminated disease and CNS disease is high**, with only one third of children having normal development after herpes encephalitis.

- **Neonates are generally not considered at risk for ACV-R by pediatricians**, and neonatal HSV is mostly the consequence of unapparent and untreated primary maternal infection with ACV-S virus.

Neonatal HSV infection

- The **HSV-2 compartmentalization** observed in our patient raises the question:

  Could the low percentage of normal neurodevelopmental outcomes observed in IV ACV-treated babies with CNS disease be due to emergence of ACV-R viruses?

- Presently, patients with HSV CNS involvement are recommended to have a lumbar puncture at the end of intravenous ACV therapy - continuation of ACV therapy is advised for patients with PCR positive CSF.

- **Viral genotyping should be considered to detect the presence of ACV-R virus** if the day 21 CSF PCR remains positive for HSV DNA or if viral CSF load raises during therapy in order to adjust treatment before neurological damage is caused.

Compartmentalization of drug-resistance

- Herpesvirus infection begins in a single compartment before spreading to others.
- After compartmentalization, differentiation continues.
- What factors may have caused compartmentalization of mutant HSV in our patient?
  - ✓ **Acyclovir uptake into the brain may be lower than in other tissues or in plasma** (PMID: 7288622) → sub-therapeutical levels of acyclovir in the brain may have resulted in the selection of TK mutated progeny during prolonged viral replication.
  - ✓ **CNS: immune privileged site**

Conclusions

- The **impact of combined ACV and PFA therapy**, followed by oral suppressive ACV therapy in the prevention of neurological sequelae, should be evaluated in a greater number of patients.

- **Early diagnosis, prompt antiviral treatment, temporal and spatial drug-resistance typing to adjust antiviral treatment, followed by suppressive ACV therapy** should be considered to avoid neurological sequelae in neonatal HSV infections.