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1 **Characterization of the dynamics of human cytomegalovirus resistance to**
2 **antiviral drugs by ultra-deep sequencing**

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27 ***Abstract***

28 Prophylactic or preemptive treatment strategies are required to prevent human
29 cytomegalovirus (CMV) infections in transplant recipients. However, treatment failure occurs
30 when CMV resistant-associated variants (RAVs) are selected. Although the diversity of CMV
31 is lower than that of RNA viruses, CMV appears to show some genetic instability, with
32 possible minor emerging resistance that may be undetectable by Sanger sequencing. We
33 aimed to examine CMV-resistance mutations over time by ultra-deep sequencing (UDS) and
34 Sanger sequencing in a kidney transplant recipient experiencing CMV infection. This patient
35 showed a transient response to three different antiviral drugs (valganciclovir, foscarnet, and
36 maribavir) and four episodes of CMV resistance over two years. The full-length UL97
37 (2.3kpb) and partial UL54 (2.4kpb) CMV genes were studied by UDS and Sanger sequencing
38 and linkage mutations calculated to determine RAVs. We detected four major and five minor
39 resistance mutations. Minor resistant variants (2 to 20%) were detected by UDS, whereas
40 major resistance substitutions (> 20%) were identified by both UDS and Sanger method. We
41 detected cross-resistance to three drugs, despite high CMV loads, suggesting that the fitness
42 of the viral mutants was not impaired. In conclusion, CMV showed complex dynamic of
43 resistance under antiviral drug pressure, as described for highly variable viruses. The
44 emergence of successive RAVs constitutes a clinically challenging complication and
45 contributes to the difficulty of therapeutic management of patients.

46

47

48 ***Introduction***

49 Human cytomegalovirus (CMV) infection is one of the most common complications affecting
50 solid organ transplant recipients, with significant morbidity and occasional mortality
51 (Ljungman et al., 2017). Thus, strategies using antiviral drugs to prevent CMV replication
52 have been recommended, allowing a significant reduction of CMV disease (Kliem et al.,
53 2008). One approach for the prevention of CMV infection is universal prophylaxis, usually
54 beginning in the immediate or very early post-transplantation period and continuing for three
55 to six months **or more** (Kotton et al., 2013). Valganciclovir (VGCV) has been demonstrated to
56 be superior to valacyclovir (VACV) **with a significant decrease in the incidence of**
57 **symptomatic disease or viremia** (Rubin et al., 2000). Despite this efficient strategy, **2.5% to**
58 **31.3%** of patients on GCV (**Ganciclovir**) prophylaxis show positive viremia following solid
59 organ transplantation (Limaye et al., 2000; Ramanan and Razonable, 2013). Various antiviral
60 drugs can be used for systemic treatment of CMV-infected patients with a compromised
61 immune status, including VGCV or GCV in the first-line, whereas cidofovir (CDV),
62 maribavir (MBV) (**not yet approved as a second line of treatment**), and foscarnet (FOS) are
63 mostly used in cases of GCV therapy failure or severe side effects.

64 Therapy failure is rare (5 to 10% of patients) and mainly due to drug-resistant CMV strains
65 that arise spontaneously under the selective pressure of antiviral treatment (Kruger et al.,
66 1999; Lurain and Chou, 2010; Singh and Yu, 1998). GCV resistance is generally due to
67 mutations in the UL97 gene, encoding viral phosphotransferase (Limaye, 2002; Young et al.,
68 2016), which **also may** affect sensitivity to MBV (**Chou, 2015a**; Chou et al., 2007). In cases of
69 severe immunosuppression and prolonged therapy, the selection of additional mutations in the
70 UL54 gene (encoding viral DNA polymerase) results in increased GCV resistance or cross-
71 resistance to CDV and/or FOS (Fischer et al., 2015).

72 The variability of CMV is debated. CMV is a DNA virus, poorly variable, with an estimated
73 variability of three to four logs less than that of RNA viruses, such as hepatitis C virus (HCV)
74 or human immunodeficiency virus (HIV) (Irwin et al., 2016). However, recent studies using
75 new technologies have shown that highly complex CMV dynamics can be observed in
76 transplant recipients (Gorzer et al., 2010; Renzette et al., 2015). These studies showed a high
77 level of mutations in envelope-associated proteins, whereas replicative enzymes, such as
78 those targeted by antiviral treatments, were mostly conserved **at least for a part of the related**
79 **genes**. According to the authors, variability also depends on the compartment considered and
80 the quality of the host immune host, suggesting a higher capacity of diversification in
81 immunocompromised patients **(Renzette et al., 2015)**. Thus, the use of deep sequencing to
82 assess such genetic variability makes it possible to better detect the emergence of CMV
83 resistance and predict and manage treatment failure (Chou et al., 2014). However, only a few
84 cases have been reported and rarely with a longitudinal follow-up (Garrigue et al., 2016).
85 Here, we describe the dynamics of CMV infection in a transplant recipient experiencing
86 multiple emergences of drug-resistance during a three-year follow-up period by ultra-deep
87 sequencing (UDS).

88

89 ***Patient and Methods***

90 ***Patient***

91 The patient was a 70-year-old woman who received a single kidney transplant for an
92 undetermined nephropathy. Multiple treatment failures were observed because of emerging
93 CMV drug-resistance mutations located within the UL97 and UL54 genes detected by Sanger
94 sequencing and UDS. The patient was seronegative for CMV, whereas her donor was
95 seropositive (D⁺/R⁻). Thus, she received three months of antiviral prophylaxis by VGCV with
96 adapted posology for renal dysfunction (450 mg/3 days). The immunosuppressive regimen

97 included prednisone, mycophenolate mofetil, cyclosporine and, later, everolimus. Various
98 antiviral drugs were used to control the CMV infections during the post-transplantation
99 period: VGCV, FOS, and MBV. Quantification of the CMV load in whole blood (Fig.1) was
100 performed every two weeks during 34 months (103 blood samples). The CMV load became
101 detectable at day 140 (D140) after transplantation, and preemptive treatment with VGCV
102 (900 mg/day) was started at D196. Despite increasing doses of VGCV, the viral load
103 remained > 4 log IU/mL and antiviral treatment was switched to FOS (5,000 mg/day). This
104 new treatment failed to reduce **sustainably** the viral load, caused edema, and was stopped 20
105 days later. As antiviral resistance to CMV was suspected, MBV (800 mg/day), a specific
106 anti-CMV drug that inhibits UL97, was introduced at D357. The CMV load decreased rapidly
107 but remained detectable (above 2.2 log IU/mL) for almost 11 months. MBV treatment was
108 replaced by VGCV (900 mg/day) at D700. As the viral load increased slowly, VGCV was
109 replaced again by MBV (800 mg/day), which finally controlled CMV replication. The viral
110 load became undetectable at D1050 and no other complication or CMV disease was reported.

111

112 *CMV load quantification*

113 Extraction from EDTA whole blood was performed using the QIAasymphony DNA mini kit
114 on a QIAasymphony (QIAGEN) instrument. A specific 105-bp region of the CMV genome
115 was amplified with CMV Artus QS-RGQ kit on a Rotor-GeneQ (QIAGEN) instrument, with
116 a detection limit of 2.2 log IU/mL.

117

118 *Extraction and PCR amplification for sequencing*

119 Whole blood samples and the International WHO standard, CMV Merlin strain (NIBSC,
120 GenBank accession no. AY446894.2), used as a control, were extracted using the
121 NUCLISENS easyMAG instrument (bioMérieux, Capronne, France). Full-length UL97

122 (2,124 bp) and UL54 (2,408 bp, nucleotides 805 to 3212) were amplified by nested PCR
123 (from 10 μ L of nucleic acid extract for PCR1 and from 5 μ L of PCR1 for PCR2) using the
124 proofreading enzyme Expand High Fidelity (Roche diagnostics) in two mixes, each
125 containing 1x Buffer, 1.5 mM MgCl₂, 800 μ M dNTP, and 300 μ M outer or inner primers
126 (UL97 First-round-PCR-outer primers [UL97PCR1SF: GTGCTCACGGTCTGGATGT ;
127 UL97PCR1ASR: CGGTGGGTTTGTACCTTCTC] ; UL97 Second-round PCR-inner primers
128 [UL97PCR2SF: GCACAACGTCACGGTACATC ; UL97PCR2ASR:
129 ACCTTCTCTGTTGCCTTTCC] ; UL54 First-round PCR-outer primers [UL54-1F:
130 ATCTGCTGGAGCAGGGTTTT ; UL54-11R: CCAATCGCTTAATGACGGCA] ; UL54
131 Second-round PCR-inner primers [UL54-2R: TTGACGGTACAGCGAGATGT ; UL54-3F:
132 GCGTCGACTTGTGATATCGA]). For both first and nested PCR, the annealing
133 temperatures were 55 °C for UL97, and 52.3 °C for UL54 as previously described
134 (Boutolleau et al., 2011; Boutolleau et al., 2009).

135

136 *Sequencing*

137 Sanger sequencing was performed with overlapping primer pairs for UL54 and UL97 using
138 the Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems,
139 Courtaboeuf, France) on an ABI 3730 Genetic Analyzer (Applied Biosystems).

140 UDS libraries were prepared for shotgun pyrosequencing according to the 454 GS Junior
141 Titanium Series Amplicon Library Preparation Method Manual using Titanium+ kits for GS
142 Junior (454 Life Sciences, Roche). Sample library quality was assessed using the Agilent
143 2100 Bioanalyzer (Agilent Technologies) before and after nebulization. DNA quantification
144 was performed using the Quant-iT PicoGreen dsDNA Assay kit (Life Technologies). DNA
145 fragments were transferred into a PicoTiterPlate (Roche) for sequencing on a GS Junior
146 instrument (Roche/454).

147

148 *Analysis*

149 Sanger sequences were analyzed using strain AD169 (GenBank accession No.BK000394) as
150 reference and SeqScape v2.5 software.

151 UDS sequences were processed using Pyropack software (inhouse software suite) (Rodriguez
152 et al., 2013). In summary, reads were demultiplexed and quality-filtered using pyroclass, the
153 sequences aligned, and variant calling performed using Pyromute. Substitution rates obtained
154 at the end of the analysis by our software were verified using QuRe (Prosperi and Salemi,
155 2012). For resistance mutations, two linkage approaches were performed, (i) using only
156 sequences covering all mutations of interest, when possible (maximum distance mutation of
157 300 bp), with PyroLink (inhouse software) (Rodriguez et al., 2015) (Rodriguez et al., 2015)
158 and/or by haplotype reconstruction with QuRe (Prosperi and Salemi, 2012).

159

160 ***Results***

161 *Sequencing performance*

162 Four blood samples (D280, D340, D357, D861) were tested by the Sanger method and eight
163 (D189, D284, D340, D343, D357, D700, D861, D903 for UL97 and D910 for UL54) by
164 UDS.

165 We obtained more than 10,000 sequences per amplicon with a mean Q score of 32.1. Merlin
166 strain sequences allowed us to establish the maximum cut-off value of mutation detection of
167 1%. The Sanger method provided no additional information over UDS (data not shown).
168 Thus, further analysis was performed using only the UDS results. **The viral sequences have
169 been uploaded to zenodo (doi: <http://doi.org/10.5281/zenodo.3428465>).**

170

171 *UL97 and UL54 resistance (Fig. 2)*

172 We identified four episodes of viral rebound (Fig. 1). Linkage of the various mutations was
173 performed to describe the dynamics of resistant-associated variants (RAVs).

174 During the D140-D310 period (VGCV), the CMV load largely rebounded, with a peak at 5.8
175 log (IU/mL). During this rebound, three RAVs were observed. The first, mono-mutated with
176 H520Q(UL97), was previously associated with GCV resistance and found to be a major
177 variant (100%) at the beginning of viral rebound. Thereafter, this viral population diversified,
178 with two additional minor resistant populations at 5% [H520Q(UL97)+K513E(UL54)] and
179 7% [H520Q(UL97)+L501F(UL54)], both resistant to GCV and CDV (Chou et al., 2014;
180 Emery, 2001; Mousavi-Jazi et al., 2001).

181 A second period of viral rebound (D330 to D370) was observed after FOS treatment
182 (administered from D290 to D330), during which the viral load increased to 4.8 log (IU/mL).

183 This rebound was associated with the emergence of two RAVS, one containing
184 [H520Q(UL97)+G841A(UL54)] at 97% and the second with one additional UL54 mutation
185 [H520Q(UL97)+G841A(UL54)+N408D(UL54)] at 3%. The mutation G841A(UL54) is
186 associated with cross-resistance to GCV, CDV, and FOS, whereas N408D(UL54) is
187 associated with only cross-resistance to GCV and FOS (Chou et al., 2014; Cihlar et al., 1998).

188 During the same period, the major variant decreased to 29% after halting FOS treatment and
189 was replaced by the mono-mutated RAV H520Q(UL97) detected at the beginning of the
190 study. At the same time, two minor RAVs emerged
191 [H520Q(UL97)+Q578H(UL54)+T700A(UL54)+G841A(UL54)] at 2% and
192 [H520Q(UL97)+Q578H(UL54)+G841A(UL54)] 3%. Both mutations, Q578H(UL54) and
193 T700A(UL54), are associated with FOS resistance (Baldanti et al., 1996; Chou et al., 2007).

194 During the third period of resistance (D650-D750) the patient was treated with MBV until
195 switching to VGCV because of viral rebound at D700. At this timepoint, UDS showed a

196 marked decrease of the H520Q(UL97) variant from 66% to 1% and the emergence of
197 [H411Y(UL97)+H520Q(UL97)+K513E(UL54)] as a major variant (94%) and a minor variant
198 [L405P(UL97)+H411Y(UL97)+H520Q(UL97)+K513E(UL54)] at 5%. Mutations
199 L405P(UL97) and H411Y(UL97) are associated with MBV resistance, suggesting cross-
200 resistance of the major variant against GCV and MBV (Campos et al., 2016; Chou, 2015b).

201 Finally, the fourth episode (D840-D950) (GCV) was associated with a viral rebound to 3 log
202 (IU/mL). The major variant of the third period
203 [H411Y(UL97)+H520Q(UL97)+K513E(UL54)] decreased to 59% and was undetectable at
204 the end of the period. Simultaneously, mono-mutated H520Q(UL97) reemerged to become
205 the major variant at the end of the study.

206

207 *Discussion*

208 Our study and several others show the capacity of UDS to detect suspected or known
209 resistance CMV mutations in immunocompromised patients (Houldcroft et al., 2016). Here,
210 we describe a very complex longitudinal case of the dynamic evolution of CMV RAVs in a
211 transplant recipient receiving three different antiviral treatments. It highlights the presence of
212 multiple viral populations composed of a major variant associated with many minor variants,
213 as observed in highly variable viruses.

214 Each antiviral drug led to the successive emergence of major resistant variants selected under
215 treatment: for VGCV, variants carrying H520Q(UL97) and K513E(UL97); for FOS, variants
216 carrying G841A(UL54); and for MBV, variants carrying H411Y(UL97). These mutations
217 have all been previously described to confer strong resistance. Mutations H520Q(UL97) did
218 not significantly impaired the fitness of the virus, as the viral load was high (above 4.5 log
219 IU/mL) during the first two periods of treatment. In addition, H520Q(UL97) seemed to have
220 little impact on fitness since this mutation persisted throughout the study without emergence

221 of UL97wt virus despite nearly a year without VGCV treatment. The viral load rebounds
222 during the third and fourth resistance periods were lower, suggesting either poor replication
223 capacity of multi-resistant variants (including K513E (UL54) and H411Y (UL97)) selected
224 over time or a concomitant antiviral activity of everolimus, as described previously
225 (Kobashigawa et al., 2013). It is also possible that the patient's immune status changes during
226 the study period with a better recognition and response against the virus allowed a better
227 control of CMV replication.

228 In parallel, many minor variants carrying supplemental resistance substitutions were co-
229 selected in this patient (L501F(UL54), N408D(UL54), Q578H(UL54), T700A(UL54),
230 L405P(UL97)). These mutations have all been previously associated with resistance. Such
231 complexity during resistance events suggests high “plasticity” of the genome during
232 replication. However, their fleeting emergence and low level suggest poor fitness of these
233 multiply-mutated variants.

234 Finally, our results also suggest the capacity of CMV to accumulate mutations through time,
235 despite periods of undetectable viral load. Indeed, mutations, such as H520Q(UL97), were
236 maintained throughout the study, possibly due to a resistance-reservoir or lack of sensitivity
237 of PCR approaches. It is also important to note that phenotypic studies including mutation
238 combinations should be tested to better understand their involvement in the level of resistance
239 and the fitness of the virus.

240 Previous CMV studies described a high level of diversity for a DNA virus but focused on
241 genes targeted by the immune system, such as the glycoprotein domain, whereas replication-
242 related genes were less variable. In the context of transplantation, with low immune pressure
243 but under antiviral treatment, our study showed a high capacity of diversification of UL54 and
244 UL97, with more than nine mutations selected during the follow-up. These results suggest

245 that CMV may harbor a high level of diversity, including replication-related genes, with
246 direct implications in treatment failure. In our case, emerging multi-resistance was observed,
247 leading to very few therapeutic options. Monitoring of resistance using highly sensitive
248 technology, such as UDS, could help to better manage transplant patients.

249

250

251

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366 **Figure 1.** CMV load (log [IU/mL]) dynamics throughout the period after transplantation. The
367 limit of detection (LOD) is indicated in red. Four periods of viral rebound were identified
368 (D140-D310, D330-D370, D660-D760, and D840-D950) and are represented as blue hatches.
369 The CMV antiviral treatments are indicated at the top in yellow for prophylactic
370 valganciclovir (vGCV), orange for curative vGCV, green for foscarnet (FOS), blue for
371 maribavir (MBV), and grey for everolimus, an immunosuppressive treatment previously
372 shown to be associated with a reduction of CMV load. CMV-resistance genotyping was
373 performed by UDS on samples indicated by the black arrows and also by Sanger sequencing
374 for those with an asterisk.

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376 **Figure 2.** Resistance-associated variant (RAV) dynamics detected by UDS in the polymerase
377 (UL54) and phosphotransferase (UL97) genes throughout the period after transplantation. The
378 viral load (in log IU/mL) is shown throughout the four periods of viral rebound (1 to 4) in the
379 background and anti-CMV antiviral treatments above the graph. The histogram shows the
380 proportion (in percent) of the RAVs. Multiple waves of RAVs, corresponding to the different

381 treatments, were detected during the four periods and included one to four different resistance
382 mutations within the viral population. This illustration shows the diversity of CMV that can
383 emerge during antiviral treatment.

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