Drug-resistant cytomegalovirus in transplant recipients: a French cohort study

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Objectives: Cytomegalovirus (CMV) drug resistance is a therapeutic challenge in the transplant setting. No longitudinal cohort studies of CMV resistance in a real-life setting have been published in the valganciclovir era. We report findings for a French multicentre prospective cohort of 346 patients enrolled at initial diagnosis of CMV infection (clinical trial registered at clinicaltrials.gov: NCT01008540).

Patients and methods: Patients were monitored for detection of CMV infection for ≥2 years. Real-time detection of resistance by UL97 and UL54 gene sequencing and antiviral phenotyping was performed if viral replication persisted for >21 days of appropriate antiviral treatment. Plasma ganciclovir assays were performed when resistance was suspected.

Results: Resistance was suspected in 37 (10.7%) patients; 18/37 (5.2% of the cohort) had virological resistance, associated with poorer outcome. Most cases involved single UL97 mutations, but four cases of multidrug resistance were due to UL54 mutations. In solid organ transplant recipients, resistance occurred mainly during primary CMV infection (odds ratio 8.78), but also in two CMV-seropositive kidney recipients. Neither CMV prophylaxis nor antilymphocyte antibody administration was associated with virological resistance.

Conclusions: These data show the feasibility of surveying resistance. Virological resistance was frequent in patients failing antiviral therapy. More than 1/5 resistant isolates harboured UL54 mutations alone or combined with UL97 mutations, which conferred a high level of resistance and sometimes were responsible for cross-resistance, leading to therapeutic failure.

Keywords: CMV, transplantation, resistance mutations, genotyping

Introduction

Cytomegalovirus (CMV) infection can have both direct and indirect detrimental effects in recipients of haematopoietic stem cell transplants (HSCT) and solid organ transplants (SOT)1,2 that can be reduced by antiviral prophylaxis and pre-emptive treatment.3,4 The emergence of CMV drug resistance, favoured by long-term exposure to antiviral drugs and by profound immunosuppression, is a growing therapeutic challenge. Resistance to ganciclovir is the most studied, because this drug (and its prodrug valganciclovir) is used as the first-line agent in ~90% of patients. Most reported resistance studies are associated with randomized therapeutic assays, with limited follow-ups. Cohort studies include a limited number of patients from single centres and are mostly
retrospective. The choice of antiviral therapy (pre-emptive or prophylactic, lasting for 3 or 6 months), as well as transplantation practices and immunosuppressive regimens, differ greatly between transplant centres. Toxicity associated with antiviral therapy increases morbidity and can lead to premature discontinuation of treatment or low doses, both of which facilitate the emergence of resistance. Our aim, therefore, was to describe the incidence, epidemiological characteristics, relative outcome of non-response to therapy and virological resistance, and various therapeutic options used in the case of resistance in a true clinical setting by analysis of a large cohort of transplant recipients. We also assessed the utility of resistance genotyping and ganciclovir assay in the management of non-responders. We conducted a prospective observational study of CMV infection in a French multicentre cohort of SOT and HSCT recipients. The study included real-time detection of resistance according to standardized criteria, and constitution of a clinical and virological database. We focused this initial report on the epidemiologic characteristics of CMV resistance and non-response to therapy.

Patients and methods

Patients

This study was conducted between September 2006 and December 2008 in 16 French transplant centres (Besançon, Bordeaux, Clermont-Ferrand, Grenoble, Lille, Limoges, Lyon, Nancy, Nantes, Paris, Reims, Rennes, Saint-Etienne, Strasbourg, Toulouse and Tours) [Prospective Multicentric Study of Cytomegalovirus Resistance in Transplant Patients and Bone Marrow Recipients (Clinical Trial Number: NCT01008540)].

Ethics

The study was approved by the human research and ethics committee of Limoges University Hospital. All patients gave their written informed consent.

Inclusion criteria

All HSCT or SOT recipients (children and adults) were prospectively enrolled on initial diagnosis of CMV infection, with or without CMV disease.

Prospective resistance survey

Monitoring of patients was envisaged for ≥2 years. Treatment and follow-up were conducted routinely in each centre. No standardization was imposed. Monitoring was based on blood CMV PCR or pp65 antigenaemia tests. When resistance was suspected, blood, urine or saliva samples were sent to the reference laboratory for virus isolation, and resistance phenotyping and genotyping.

Definitions

CMV infection/disease were defined according to the American Society of Transplantation recommendations on screening, monitoring and reporting of infectious complications in immunosuppression trials in recipients of organ transplantation. 5

CMV infection

Evidence of CMV replication was diagnosed from viral culture, the presence of intracytoplasmic or intranuclear inclusions, CMV immunohistochemistry in histological sections, or viral nucleic acid or antigen assays, regardless of symptoms. The threshold defining CMV replication in blood was 1000 DNA copies/mL or two consecutive values of >600 copies/mL for in-house or commercial real-time PCR assays, and more than one pp65-positive blood leucocyte test for antigenaemia.

CMV disease

Evidence of CMV infection with symptoms, classified as CMV syndrome, was recorded if any of the following was found: fever >38°C for ≥2 days; new or increased malaise; leucopenia; ≥5% atypical lymphocytes; thrombocytopenia; hepatic transaminase elevation (ALT or AST) more than double the upper normal limit (non-liver transplant recipients only); and symptoms associated with CMV-positive blood culture, antigenaemia or DNA/RNA positivity, and no other identified cause of the symptoms and signs. A recording of tissue-invasive disease was made if the following were present: symptoms and signs of tissue invasion; and CMV-positive biopsy specimen (e.g. pneumonitis, hepatitis, retinitis and gastrointestinal disease).

Prophylaxis

Anti-CMV therapy administered to at-risk patients before detection of CMV infection.

Pre-emptive therapy

Treatment administered to patients with CMV infection, with the aim of preventing the CMV syndrome and CMV disease.

Curative therapy

Treatment administered for the CMV syndrome or CMV disease.

Recurrent CMV infection

CMV infection (symptomatic or asymptomatic) occurring ≥3 months post-transplantation in patients after proven eradication of previous active CMV infection at the end of treatment 2 (in practice, two negative samples separated by ≥1 week).

Suspected resistance

Persistent viral replication, regardless of clinical manifestations, after ≥3 weeks of appropriate antiviral treatment (ganciclovir, valganciclovir, foscamet or cidofovir as curative treatment, or ganciclovir, valganciclovir or valaciclovir as prophylaxis, at doses recommended by the manufacturer).

Clinical drug resistance

Persistent viral replication in the absence of virological resistance.

Virological resistance

Detection of a resistant CMV by genotypic or phenotypic resistance testing.

Genotypic and phenotypic resistance testing

Genotypic and phenotypic testing were performed as routine analyses. Resistance genotype results were reported to clinicians between 4 and 7 days. Phenotype results were delayed because of the need for growth
of the strain. After virus isolation, phenotypic susceptibility to ganciclovir, cidofovir and foscarnet was tested using the Agence Nationale de Recherche sur le Sida (ANRS) consensus method (a standardized 24-well-plate focus reduction assay) and/or by real-time PCR assay. Maribavir resistance phenotyping was performed by focus reduction assay, as described previously.

Phenotypic resistance was defined using a sensitivity index (SI), which is the IC50 (drug concentration that inhibits 50% of virus growth) of the isolate divided by the IC50 of reference strain AD169, tested in parallel. Isolates were considered as resistant if their SI was >3 (the IC50 thus being >3 × that of the reference strain).

Genotyping was based on analysis of UL97 and UL54 mutations. Kinase UL97 allows ganciclovir or aciclovir activation by phosphorylation and is the target of maribavir. UL54 is the target of current antiviral drugs.

100 μL of patient serum spiked with 50 μL of aciclovir (internal standard). The calibration curves were linear from 10 to 10000 μg/L (r² > 0.99). For concentrations >10000 μg/L, dilutions were performed. The within-day and between-day coefficients of variation were <8.8% over this range. Ganciclovir calibration standards at 0, 10, 50, 100, 500, 1000, 5000 and 10000 μg/L were prepared, extracted and analysed with each series, together with internal quality controls at three different concentrations. In accordance with previously reported pharmacodynamic data and to the IC50 (2.5–6 μM) for wild-type CMV isolates in the focus or plaque reduction assay and the IC50 of 0.56 mg/L (2.24 μM) to 2 mg/L (8 μM).

Plasma ganciclovir assay
When resistance was suspected, a plasma sample was collected at baseline exposure and stored at −80°C. Ganciclovir was assayed in a central laboratory with a validated, specific, reverse-phase high-performance liquid chromatography–tandem mass spectrometry (LC–MS/MS) method with a 2000 QTRAP LC–MS/MS system (Applied-Biosystem/Sciex, Foster City, CA, USA). Sample preparation involved protein precipitation from 100 μL of patient serum spiked with 50 μL of aciclovir (internal standard). The calibration curves were linear from 10 to 10000 μg/L (r² > 0.99). For concentrations >10000 μg/L, dilutions were performed. The within-day and between-day coefficients of variation and bias were <8.8% over this range. Ganciclovir calibration standards at 0, 10, 50, 100, 500, 1000, 5000 and 10000 μg/L were prepared, extracted and analysed with each series, together with internal quality controls at three different concentrations. In accordance with previously reported pharmacodynamic data and to the IC50 (2.5–6 μM) for wild-type CMV isolates in the focus or plaque reduction assay and the IC50 of 0.56 mg/L (2.24 μM) to 2 mg/L (8 μM).

Data collection and analysis
The type of transplant, donor and recipient CMV serostatus, indication for transplantation, antiviral prophylaxis (type, dose and duration), antiviral treatments (type, dose and duration), immunosuppressive regimen, CMV load and clinical status were collected at inclusion and when resistance was suspected. Donor and recipient CMV-IgG serostatus were determined at the time of transplantation in each centre using serological tests approved by the French Health Authorities.

Data were analysed with descriptive statistical methods.

Results
Study population
From September 2006 to December 2008, 443 allograft patients were enrolled. Mean follow-up duration was 993 days (493–1493 days; standard deviation 707). Virological monitoring consisted of PCR testing for blood CMV (87.8%) and antigen assay (12.2%). Ninety-seven patients did not receive any antiviral drug and were therefore excluded from the analysis. The study population thus consisted of 346 patients, including 287 SOT (224 kidney, 28 heart, 24 liver, 7 lung and 4 multiorgan) and 59 HSCT recipients. When compared with the treated patients, the 97 untreated patients were mostly kidney recipients (87.6% versus 64.7%), less CMV seropositive donor (D+)/seronegative recipients (R−) (5.1%, 5/97) and had lower viral load (<3 log10 copies/mL in most untreated patients). Among the 346 included patients, in SOT recipients (n = 287), donor/recipient CMV serostatus was mainly D+/R− (n = 119, 41.5%), with fewer D+/R+ (n = 111, 38.7%) and D−/R+ (n = 50, 17.4%). In HSCT recipients (n = 59), CMV serostatus was mainly D−/R+ (n = 27, 45.7%). Nine patients (seven SOT recipients and two HSCT recipients) were registered D−/R− at the time of transplantation. Concerning prophylaxis, 198 patients (57.2%) received ganciclovir (58%, n = 115), valganciclovir (36.5%, n = 72), aciclovir (3.5%, n = 7), intravenous ganciclovir (1.5%, n = 3) or maribavir (0.5%, one liver transplant recipient) (Figure 1). Five D−/R− recipients received valganciclovir (three SOT) or Herpes simplex virus (HSV) prophylaxis with valaciclovir (two HSCT). The median time between transplantation and initial diagnosis of CMV infection was 128 days (6–465 days) for patients who received prophylaxis and 35 days (3–548 days) for patients without prophylaxis. Of the 198 patients receiving prophylaxis, 58 experienced their first CMV infection during prophylaxis (24/118 under ganciclovir or valganciclovir, 32/72 under valaciclovir, 2/7 under aciclovir).

Virological resistance
Virological resistance was documented in any patient with suspected resistance, whether they received ganciclovir, valganciclovir or any other treatment (see Tables 2 and 3 for virological pharmacological and other patient data).

Resistance was suspected in 10.7% (37/346) of patients, including 33/287 (11.5%) SOT and 4/59 (6.8%) HSCT recipients. The SOT subpopulation included 23/224 (10.3%) kidney, 2/28 (7.1%) heart, 3/24 (12.5%) liver, 5/7 (71.4%) lung and 0/4 (0%) multiorgan recipients. Eighteen (48.6%) of the 37 patients with suspected resistance (5.2% of the 346 patients of the cohort) had virological resistance, including 17/287 SOT recipients (5.9%) and 1/59 HSCT recipient (1.7%). The SOT subpopulation included 14/224 (6.2%) kidney, 2/24 (8.4%) liver and 1/7 (14.3%) lung recipients. The overall incidence of resistance was 4.12 cases per 100 patient-years. The incidence of resistance was 3.76 cases per 100 patient-years in the subgroup receiving...
prophylaxis \( n = 198 \) and 4.62 cases per 100 patient-years in the subgroup receiving pre-emptive or curative therapy \( n = 148 \). All 18 recipients with virological resistance had received ganciclovir (or valganciclovir) at least once and 9 patients had received prophylaxis with valganciclovir (6/9) or valaciclovir (3/9).

**Clinical manifestations at onset of suspected resistance**

Resistance was suspected a median of 187 days post-transplantation (43–700 days) or 39 days (7–627 days) after initial CMV infection in patients with clinical resistance. In patients with confirmed virological resistance, resistance was suspected a median of 182 days post-transplantation (100–468 days) or 46 days (11–231 days) after initial CMV infection. The median cumulative treatment duration before the onset of clinical resistance was 156 days (range 17–426 days) and 151 days (33–360 days) before virological resistance, respectively. No significant difference in clinical manifestations was noted between patients with clinical drug resistance and virological resistance.

Initial CMV infection was associated with symptoms in 14 of the 37 cases of suspected resistance (6 colitis, 1 encephalitis and 7 CMV syndromes), of whom 6 had virological resistance and 8 had clinical drug resistance.

**Plasma ganciclovir concentrations**

Ganciclovir concentration was assayed when resistance was suspected in 16 patients, 9 in the clinical drug resistance group and 7 in the virological resistance group. There was substantial dispersion of ganciclovir concentrations, possibly due to the diversity of renal function in these patients (renal clearances are not currently available). Ganciclovir concentrations were very low (<0.6 \( \mu \)M) in three patients, two without virological resistance and one with virological resistance [Patients 141 and 66 (Table 1), and 164 (Table 2)].

**Consequences of genotyping in clinical practice**

Among the 19 patients with clinical resistance, therapeutic adaptation (known for 14/19 patients) was reduced immunosuppressive therapy (5/14), higher ganciclovir doses (2/14), a switch to foscarnet despite the absence of resistance (3/14) or no modification (4/14).

Among the 18 patients with virological resistance, therapy was adapted to the results of genotyping by changing the antiviral molecule in 16/18 cases, with lowering immunosuppression in 9/18 cases. Therapeutic adaptation is detailed in Tables 1 and 2.

**Comparative outcomes for patients with clinical resistance and virological resistance**

Clinical outcome was favourable in 11/16 cases (69%) with clinical resistance (data not provided in 3 cases), and CMV viral load became undetectable in 15/16 patients (94%), including those with low plasma ganciclovir levels. One patient (lung transplant recipient) developed CMV colitis and subsequently died from bacterial endocarditis. Four patients developed complications not directly related to CMV infection (two graft-versus-host disease, one antibody-mediated renal graft rejection and one death from acute lymphoid leukaemia). Treatment data were collected for 16 patients: reduction in immunosuppressive therapy (5/16);...
Table 1. Description of the 19 patients with clinical drug resistance (no detected mutations in UL97 and UL54)

<table>
<thead>
<tr>
<th>Patient</th>
<th>Tx</th>
<th>CMV antibody status (D/R)</th>
<th>Onset of CMV infection from Tx (days)</th>
<th>Onset of clinical resistance from Tx (days)</th>
<th>Symptomatic at time of clinical resistance</th>
<th>Prophylaxis</th>
<th>Treatment before suspected resistance</th>
<th>Plasma GCV (µM)³ (drug administered)</th>
<th>Therapeutic adaptation</th>
<th>Virological and clinical outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>lung</td>
<td>D+/R+</td>
<td>268</td>
<td>NA</td>
<td>700</td>
<td>NA</td>
<td>VGCV</td>
<td>230</td>
<td>VGC, VGCV</td>
<td>397</td>
</tr>
<tr>
<td>22</td>
<td>lung</td>
<td>D+/R−</td>
<td>465</td>
<td>abdominal pains</td>
<td>467</td>
<td>persistence of abdominal pains</td>
<td>VGCV</td>
<td>424</td>
<td>GCV, VGCV</td>
<td>2</td>
</tr>
<tr>
<td>23</td>
<td>kidney</td>
<td>D+/R−</td>
<td>90</td>
<td>none</td>
<td>104</td>
<td>none</td>
<td>VGCV</td>
<td>106</td>
<td>GCV</td>
<td>13</td>
</tr>
<tr>
<td>24</td>
<td>heart</td>
<td>D+/R+</td>
<td>13</td>
<td>none</td>
<td>53</td>
<td>none</td>
<td>none</td>
<td>0</td>
<td>GCV</td>
<td>22</td>
</tr>
<tr>
<td>38</td>
<td>kidney</td>
<td>D+/R−</td>
<td>272</td>
<td>fever</td>
<td>313</td>
<td>fever, neutropenia</td>
<td>VGCV</td>
<td>144</td>
<td>GCV</td>
<td>23</td>
</tr>
<tr>
<td>66</td>
<td>HSCT</td>
<td>D+/R+</td>
<td>113</td>
<td>CMV colitis</td>
<td>135</td>
<td>none</td>
<td>none</td>
<td>0</td>
<td>GCV+FOS</td>
<td>17</td>
</tr>
<tr>
<td>87</td>
<td>kidney</td>
<td>D+/R−</td>
<td>67</td>
<td>none</td>
<td>342</td>
<td>none</td>
<td>VGCV</td>
<td>79</td>
<td>VGCV</td>
<td>141</td>
</tr>
<tr>
<td>95</td>
<td>lung</td>
<td>D+/R−</td>
<td>129</td>
<td>none</td>
<td>296</td>
<td>none</td>
<td>VGCV</td>
<td>228</td>
<td>VGCV</td>
<td>55</td>
</tr>
<tr>
<td>105</td>
<td>kidney</td>
<td>D+/R+</td>
<td>14</td>
<td>none</td>
<td>113</td>
<td>none</td>
<td>none</td>
<td>0</td>
<td>VGCV</td>
<td>31</td>
</tr>
</tbody>
</table>
| Tx, transplant; D/R, donor/recipient; ALL, acute lymphoid leukaemia; HSCT, haematopoietic stem cells transplant; GVHD, graft-versus-host disease; CMV, cytomegalovirus; CMV viraemia neg, means that DNAemia or antigenaemia became negative either spontaneously or after therapeutic modification; favourable clinical outcome, means no further clinical events attributable to CMV infection; VCV, valaciclovir; GCV, ganciclovir; VGCV, valganciclovir; FOS, foscarnet; MMF, mycophenolate mofetil; CS, corticosteroids; iv Ig, intravenous immunoglobulins; d, day(s); ND, not done; NA, clinical information not available; GCV dosage, residual GCV plasma concentrations (values in parentheses are the doses given to the patients).
<p>| aResidual GCV assays indicated in bold were performed simultaneously with resistance testing. |</p>
<table>
<thead>
<tr>
<th>Patient</th>
<th>Tx</th>
<th>CMV serostatus (D/R)</th>
<th>Onset of CMV infection from Tx (days)</th>
<th>Symptoms</th>
<th>Onset of CMV infection from Tx (days) ([1]/[2])</th>
<th>Prophylaxis drugs (1st line, 2nd line, etc.)</th>
<th>Treatment before resistance</th>
<th>Proved resistance mutation detected in Plasma GCV (μM) (drug administered)</th>
<th>Therapeutic adaptation</th>
<th>Virological and clinical outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>41</td>
<td>liver</td>
<td>D+/R−</td>
<td>258</td>
<td>fever, loss of weight</td>
<td>468</td>
<td>VGCV</td>
<td>225</td>
<td>GCV, VGCV, GCV, VGCV</td>
<td>135</td>
<td>+</td>
</tr>
<tr>
<td>83</td>
<td>HSCT</td>
<td>D−/R+</td>
<td>35</td>
<td>CMV colitis</td>
<td>118</td>
<td>VCV</td>
<td>31</td>
<td>VCV, VCC, VCV, GCV</td>
<td>66</td>
<td>+</td>
</tr>
<tr>
<td>97</td>
<td>kidney</td>
<td>D+/R−</td>
<td>76</td>
<td>none</td>
<td>256</td>
<td>VCV</td>
<td>80</td>
<td>VCC, VCC, GCV</td>
<td>110</td>
<td>+</td>
</tr>
<tr>
<td>114</td>
<td>kidney</td>
<td>D+/R−</td>
<td>30</td>
<td>none</td>
<td>197</td>
<td>none</td>
<td>0</td>
<td>VCC, VCC, GCV</td>
<td>192</td>
<td>+</td>
</tr>
<tr>
<td>152</td>
<td>kidney</td>
<td>D+/R−</td>
<td>37</td>
<td>none</td>
<td>128</td>
<td>none</td>
<td>0</td>
<td>VCC, VCC, VCC</td>
<td>85</td>
<td>+</td>
</tr>
<tr>
<td>157</td>
<td>liver</td>
<td>D+/R−</td>
<td>211</td>
<td>none</td>
<td>312</td>
<td>VGCV</td>
<td>184</td>
<td>GCV</td>
<td>104</td>
<td>+</td>
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<tr>
<td>164</td>
<td>kidney</td>
<td>D+/R−</td>
<td>24</td>
<td>fever, neutropenia</td>
<td>295</td>
<td>none</td>
<td>0</td>
<td>VCC, VCC, VCC</td>
<td>118</td>
<td>+</td>
</tr>
<tr>
<td>189</td>
<td>kidney</td>
<td>D+/R+</td>
<td>32</td>
<td>none</td>
<td>144</td>
<td>none</td>
<td>0</td>
<td>VCC, GCV</td>
<td>66</td>
<td>+</td>
</tr>
<tr>
<td>202</td>
<td>kidney</td>
<td>D+/R−</td>
<td>16</td>
<td>none</td>
<td>247</td>
<td>none</td>
<td>0</td>
<td>VCC, GCV</td>
<td>143</td>
<td>+</td>
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<tr>
<td>Patient ID</td>
<td>Organ</td>
<td>Diagnosis</td>
<td>Site</td>
<td>CMV Load</td>
<td>Treatment</td>
<td>Viral Load Persistence</td>
<td>Clinical Outcome</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>------------</td>
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<td></td>
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</tr>
<tr>
<td>210 Kidney</td>
<td>D+R+</td>
<td>Diarrhoea</td>
<td>120</td>
<td>None</td>
<td>VGCV, GCV</td>
<td>67</td>
<td>FOS 6 g/d, 29 d, then 8 g/d, 15 d</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>VGCV, GCV, GCV</td>
<td></td>
<td>Weak viral load persistence after end of FOS; favourable clinical outcome</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>224 Kidney</td>
<td>D+R-</td>
<td>None</td>
<td>126</td>
<td>None</td>
<td>VCV, GCV</td>
<td>43</td>
<td>FOS 1 g/d, 43 d, then GCV 400 mg/d, 108 d</td>
<td></td>
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<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>GCV, FOS</td>
<td></td>
<td>No change in antiviral therapy</td>
<td></td>
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<tr>
<td>240 Kidney</td>
<td>D+R-</td>
<td>None</td>
<td>168</td>
<td>None</td>
<td>VGCV, FOS</td>
<td>42</td>
<td>FOS 1 g/d, 43 d, then GCV 400 mg/d, 108 d</td>
<td></td>
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<td></td>
<td>VGCV, GCV</td>
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<td>No change in antiviral therapy</td>
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<tr>
<td>263 Kidney</td>
<td>D+R-</td>
<td>Fever,</td>
<td>101</td>
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<td>VGCV, GCV</td>
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<td>FOS 1 g/d, 43 d, then GCV 400 mg/d, 108 d</td>
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<td>Leucopenia</td>
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<td>No change in antiviral therapy</td>
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<td>269 Lung</td>
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<td>77</td>
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<td>VGCV, GCV</td>
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<td>VGCV, GCV</td>
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<td>Favourable clinical outcome</td>
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<td>438 Kidney</td>
<td>D+R-</td>
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**Legend:**
- **Tx:** transplant
- **D/R:** donor/recipient
- **HSCT:** haematopoietic stem cells transplant
- **GVHD:** graft-versus-host disease
- **VCV:** valaciclovir
- **GCV:** ganciclovir
- **VGCV:** valganciclovir
- **FOS:** foscarnet
- **CDV:** cidofovir
- **MMF:** mycophenolate mofetil
- **CS:** corticosteroids
- **iv Ig:** intravenous immunoglobulins
- **d:** day(s)
- **CMV viraemia neg:** DNAemia or antigenaemia became undetectable either spontaneously or after therapeutic modification
- **favourable clinical outcome:** no further clinical events attributable to CMV infection
- **ND:** not done
- **NA:** clinical information not available
- **S1:** first sample with a resistance mutation
- **S2:** second sample with another resistance mutation
- **(–):** newly described mutations, found in patients who were non-responders to treatment; they have not yet been identified by marker transfer experiments as resistance mutations
- **Residual GCV assays indicated in bold were performed simultaneously with resistance testing.**

---

**Notes:**
- Cytomegalovirus resistance in transplantation
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**Additional Notes:**
- Residual GCV assays indicated in bold were performed simultaneously with resistance testing.
- Dual-agent therapy for 56 days.
higher ganciclovir doses (3/16); switch to foscarnet despite the absence of resistance (3/16); or no modification (5/16).

Clinical outcome was available in 16/18 cases with virological resistance. It was favourable in 7/16 cases (43.75%), but with persistent viraemia in 3 cases, and unfavourable in 9/16 patients (56.25%), associated with either isolated persistent viraemia in 1 case or severe diseases in 8 cases (loss of kidney allograft (2/8, 1 because of CMV disease); death (4/8, 1 from metastatic spino-cellar cancer and 3 from CMV pneumonia); and nephropathy due to BK virus (2/8)).

**UL97 and UL54 gene analysis (Table 3)**

Of the 18 patients with virological resistance, 17 (94.4%) harboured CMV variants with resistance mutations in gene UL97. High-level resistance mutations M460V (3/17), A594V (2/17), L595S (6/17), L595F (2/17) and a 12 codon deletion (del591–603), and low-level resistance mutations C592G, A594P and C603W (in one patient each) were found.14,17 None of these patients had received maribavir and none had maribavir resistance mutations.14,17 Five UL54 resistance mutations were confirmed: N408K,18 D413E19 and A987G, 20 conferring cross-resistance to ganciclovir and cidofovir; L802M, conferring cross-resistance to ganciclovir and foscarnet; 19 and A834P, conferring multidrug resistance. 20 Two novel mutations were observed: D515Y, mapping to the same position as both the D515E resistance mutation 21 and the substitution D515G, which is not associated with resistance;19 and A614S, mapping to a non-conserved region.22

UL97 mutations were associated with UL54 mutations in four CMV variants from three patients. Patient 438, a CMV-seronegative kidney recipient, gave two sequential samples with resistant strains harbouring different UL54 mutations. The first isolate was recovered 120 days post-transplantation, only 5 days after ganciclovir initiation, but after a cumulative treatment period of 91 days (86 days of valganciclovir prophylaxis and 5 days of ganciclovir therapy); this isolate harboured a UL97 mutation (C592G) and a UL54 mutation (A987G). The second isolate was recovered 216 days post-transplantation, after a cumulative treatment period of 174 days (15 additional days of ganciclovir therapy and 68 days of foscarnet therapy); this isolate harboured the same UL97 mutation (C592G) and another UL54 mutation (N408K). Sequencing provided no evidence of a dual viral population. Patient 83, an HSCT recipient, and Patient 263, a kidney recipient, gave only one isolate each, harbouring both UL54 and UL97 mutations.

The multidrug resistance UL54 mutation A834P was detected alone in a CMV-seropositive kidney recipient (Patient 202), leading to transplantectomy.

**Resistance phenotypes and susceptibility to maribavir**

CMV isolates from six clinical samples were tested for antiviral susceptibility. The phenotypes were consistent with the

<table>
<thead>
<tr>
<th>Patient</th>
<th>Tx</th>
<th>Drugs used</th>
<th>Exposure to antiviral compounds duration (days)</th>
<th>Detected mutation</th>
<th>Genotypic resistance</th>
<th>Phenotypic resistance</th>
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<tr>
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<td>UL97</td>
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<td>41</td>
<td>liver</td>
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<td>HSCT</td>
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<td>97</td>
<td>M640V</td>
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<td>114</td>
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<td>L595S</td>
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<td>C603W</td>
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<td>67</td>
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<tr>
<td>224</td>
<td>kidney</td>
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<td>189</td>
<td>M460V</td>
<td>absence</td>
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<td>L595F</td>
<td>D413E</td>
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<td>lung</td>
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<td>33</td>
<td>L595S</td>
<td>absence</td>
<td>R S S</td>
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<td>346</td>
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<td>146</td>
<td>L595F</td>
<td>absence</td>
<td>R S S</td>
</tr>
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<td>403</td>
<td>kidney</td>
<td>GCV or VGCV</td>
<td>60</td>
<td>A594V</td>
<td>absence</td>
<td>R S S</td>
</tr>
<tr>
<td>438</td>
<td>kidney</td>
<td>VGCV, GCV, FOS</td>
<td>91 (S1)</td>
<td>C592G (S1)</td>
<td>A987G (S1)</td>
<td>R S S</td>
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<td></td>
<td></td>
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<td>174 (S2)</td>
<td>C592G (S2)</td>
<td>N408K (S2)</td>
<td>R S S</td>
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</table>

Tx, transplant; HSCT, haematopoietic stem cell transplant; VCV, valaciclovir; GCV, ganciclovir; VGCV, valganciclovir; FOS, foscarnet; R, resistant; S, susceptible; ND, not done; NS, no strain available; S1, first sample with a resistance mutation; S2, second sample with another resistance mutation; ?, resistance profile not yet described; CDV, cidofovir; CMV, cytomegalovirus.

Mutations in italics and bold are newly described mutations, found in patients who were non-responders to treatment; they have not yet been identified by marker transfer experiments as resistance mutations.

Table 3. Resistance mutations detected in UL97 and UL54 after anti-CMV drugs exposure with their genotypic and phenotypic resistance profiles
genotypes, except for the two isolates from Patient 438; the first isolate displayed resistance to ganciclovir (SI_{50}=10), slightly reduced susceptibility to cidofovir (SI_{50}=2) and was susceptible to foscarnet. The second isolate was resistant to ganciclovir and cidofovir (SI_{50}=10 for both drugs), with a small reduction in susceptibility to foscarnet (SI_{50}>1). This was unexpected and may have been due to variability in the foscarnet phenotypic assay. These isolates are being further characterized. The two isolates harbouring UL97 mutations associated with new UL54 mutations (DS15Y and A614S) were both resistant to ganciclovir only, so there was no evidence that these new mutations contributed to CMV resistance. The IC_{50} values of maribavir were close to that of the reference strain for all six isolates (SI_{50}<3), indicating maribavir susceptibility.

**Antiviral resistance and non-response to therapy**

Primary infection was a major risk factor for virological resistance in SOT recipients, with an odds ratio (OR) of 8.78 [95% confidence interval (CI) 2.49–30.95, P value 6.6×10^{-5}] (as illustrated in Figure 2). Two of the 18 patients (Patients 189 and 210) with virological resistance and unfavourable outcome were CMV-seropositive kidney recipients. One of them, Patient 189 (D+/R+) received a kidney graft in 2006, but had received a lung graft in 1992. This patient has been exposed to previous antiviral treatment (36 days of GCV) that could have exerted selection pressure, and therefore was more susceptible to the emergence of resistance. For Patient 210, confirmed D+/R+, we have no more information that might explain the emergence of the resistance mutation. CMV prophylaxis (aciclovir, valaciclovir or valganciclovir or ganciclovir) was not significantly associated with virological resistance [OR=0.74 (95% CI 0.27–1.98, P value 0.55) for prophylaxis versus pre-emptive treatment] in SOT recipients. The corresponding OR could not be calculated for HSCT recipients, because none of those who did not receive prophylaxis developed resistance mutations. Antilymphocyte antibody therapy at the time of induction of immunosuppression (nine patients with virological resistance and nine patients with clinical drug resistance) was not a risk factor for virological resistance [OR=0.83 (95% CI 0.32–2.15, P value 0.70)]. Neither was the mean duration of exposure to antiviral drugs [151 days (range 33–360 days) in the virological resistance group and 156 days (range 17–424 days) in the clinical drug resistance group].

The median viral load measured at the onset of CMV infection was 3.51 log_{10} DNA copies/mL (2–8.4 log_{10} DNA copies/mL) in the 293 patients without suspected resistance, 3.76 log_{10} DNA copies/mL (2.3–5.4 log_{10} DNA copies/mL) in the 19 patients with clinical resistance and 3.77 log_{10} DNA copies/mL (3.2–6.7 log_{10} DNA copies/mL) in the 18 recipients with confirmed virological resistance, and thus was not associated with the risk of persistent viraemia.

**Discussion**

This study of CMV resistance in transplant patients is, to our knowledge, the first prospective real-life survey reported in the era of valganciclovir. We opted for a multicentre study, to cover the diversity of clinical and virological practices across French centres. Resistance was suspected when viral replication persisted after 3 weeks of treatment. This period was chosen because studies of CMV kinetics showed that the viral load drops below the detection threshold by day 21 of ganciclovir treatment in most patients. Single testing at day 15 may lead to false-negative results. Recent published results from Boivin et al. and Asberg et al. confirmed that this choice was relevant. Persistent viraemia at day 21 of therapy, but not at day 14, was shown to be a risk factor for the emergence of resistance. Moreover, the development of resistance during treatment was associated with failure to eradicate CMV DNAemia in plasma, both at day 21 and at day 49. Genotypic testing was carried out in real time; the results combined with counselling were expected to be helpful for clinicians in their routine practice. The UL97 and UL54 genes were completely sequenced to detect both known and novel drug resistance mutations, using a published method routinely used in the two reference centre laboratories. We report our initial results for the period September 2006 to December 2008, based on descriptive statistics and focusing on cases of resistance.

The overall incidence of CMV resistance was lower than in cohort studies conducted before 2001 in SOT recipients (5.9% versus 9.5%). As in these previous cohorts, lung transplant recipients tended to have a higher risk, but were few in number. Recent studies report ganciclovir resistance rates of 1.5% for heart transplant recipients and of 3.6%–6.2% in SOT recipients overall, rates similar to those in our cohort. The data from our observational cohort are consistent with data from single- and multi-centre controlled studies, suggesting that the findings are pertinent to clinical practice in general. No large studies of resistance in HSCT recipients have been published previously. Resistance was rare in our HSCT transplant population, occurring in only one of 59 patients (1.7%), compared with 7.6% (1/13) in the study by Allice et al.

In our cohort, virological resistance was confirmed in half of the cases of suspected resistance. Therefore, other factors contribute to the emergence of resistance.

**Figure 2.** Distribution of patients with virological and clinical resistance, according to donor/recipient CMV serostatus. D+, donor; R−, recipient; CMV, cytomegalovirus.
to treatment failure, as previously suggested. Nevertheless, the proportion of patients in our cohort who suffered treatment failure in the absence of antiviral resistance, although high, was much lower than that described recently in a small cohort of D+/-R− kidney recipients. Persistent CMV replication during valganciclovir or intravenous ganciclovir therapy raises the risk of resistance, underlining the usefulness of guidelines for resistance testing. The clinical outcome of the non-responders to therapy according to the presence or absence of antiviral resistance remains controversial. Longitudinal cohort studies may resolve this issue. In our study, persistent viraemia was more frequent in cases of virological resistance and was associated with either CMV disease or opportunistic diseases. Genotyping, based on standardized criteria for early virological resistance detection, is therefore valuable to prevent both unfavourable outcomes and the emergence of multidrug resistance.

Contrasting with studies published to date showing that most ganciclovir-resistant strains have had mutations in UL97 alone, 22.2% of our resistant isolates harboured UL54 mutations alone or combined with UL97 mutations; these mutations confer higher levels of phenotypic resistance to ganciclovir, cross-resistance to cidofovir and, less likely, to foscarnet, and can cause late-onset disease with multidrug resistance. Note that one mutant with an isolated UL54 mutation A834P, responsible for cross-resistance to all antiviral drugs, emerged in a seronegative kidney recipient (undergoing his third graft) receiving both ganciclovir and foscarnet. This suggests that this drug combination should be used with caution in pre-treated patients, especially if both drugs are given at low doses. The mean duration of treatment (151 days) before the emergence of virological resistance was consistent with mathematical modelling by Emery et al. Interestingly, the time to onset of clinical resistance was very similar, suggesting that resistant strains are as fit as non-resistant strains and that antiviral drugs unmask pre-existing minority mutants.

The main risk factor for the emergence of resistant viruses in our cohort was D+/-R− serostatus, as previously reported. Resistance to ganciclovir has been described in CMV-seropositive lung transplant recipients. However, resistance can also occur in seropositive organ recipients, as was the case for 2 of our 17 SOT recipients. The emergence of resistance was not associated with viral load level at diagnosis of CMV infection, the duration of therapy or the use of antilymphocyte antibodies at induction. Inadequate ganciclovir doses can induce clinical resistance and favour the emergence of resistance-associated mutations within UL97 and, sometimes, UL54. Moreover, optimal ganciclovir doses can overcome virological resistance due to isolated UL97 resistance mutations, at least for a certain period. Recent studies have shown that valganciclovir pharmacokinetic variables remain stable and similar in patients when treatment is adjusted to renal function and body weight. Most of our patients were exposed to ganciclovir concentrations close to the IC₅₀ values for clinical isolates, although the variability of the plasma ganciclovir concentration suggests that adaptation to renal function and body weight was not satisfactory. The usefulness of measuring plasma ganciclovir concentrations in patients with fluctuating renal function, together with genotyping to avoid unnecessary changes in antiviral therapy and, thus, unnecessary additional toxicity should be further explored.

Our study has some limitations linked to the heterogeneity of the cohort. Diverse treatments were administered for the prevention of CMV infection with respect to the serostatus. Consequently, there are large numbers of small subgroups and pairwise univariate/multivariate comparisons are difficult. However, the prospective design, the standardized criteria defining antiviral and clinical drug resistance, the large number of patients, and the real-life survey could offer complementary insight into current recommendations in both the SOT and HSCT settings. We also report the first cohort data on HSCT recipients.

In conclusion, these preliminary results show that CMV resistance remains a problem in transplant recipients, in routine clinical settings, in the prophylaxis era. Multiple drug resistance caused by UL54 mutations is not exceptional. Alternative antiviral drugs are therefore needed, and standardized genotyping of both UL97 and UL54 genes (and possibly ganciclovir assays) can be useful for adapting treatment. Guidelines for taking care of patients infected by resistant CMV are clearly needed and such cohorts with standardized criteria for resistance could be useful for measuring the impact of current recommendations in clinical practice.

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Transparency declarations

None to declare.

Author contributions

S. H. participated in performance of the research by routine virological follow-up, data collection and global analysis of data, statistical analysis, resistance follow-up and writing of the manuscript. He also participated in routine virological follow-up, patient recruitment based on virological criteria and providing virological data, samples and isolates for the Limoges centre. F. G.-G. participated in research design and performance of the research by virological and clinical data collection for all centres. She organized and managed the data bank. M.-C. M. participated in research design, performance of the research as a reference laboratory for Resistance Survey by resistance follow-up (genotyping, phenotyping and counselling) for the participating centres from the northern part of France and writing of the manuscript. I. G. participated in performance of the research by routine virological follow-up, patient recruitment based on virological criteria, providing virological data and writing of the manuscript. P. M. participated in performance of the research by patient recruitment, providing clinical data and writing of the manuscript. C. M. participated in performance of the research by routine virological follow-up, patient recruitment based on virological criteria and providing virological data, samples and isolates. L. R. participated in performance of the research by patient recruitment, providing clinical data and giving helpful advice on research design. F. S. M. participated in performance of the research by dosing ganciclovir. M. E. participated in performance of the research by patient recruitment, providing clinical data, design of the data bank and writing of the manuscript. J.-P. R. participated in performance of the research by patient recruitment and providing clinical data. S. C. participated in performance of the research by being responsible for antiviral assays. R. G. participated in performance of the research by routine virological follow-up, patient recruitment based on virological criteria and providing virological data, samples and isolates. S. P. participated in performance of the research by patient recruitment and providing clinical data. S. A. designed and coordinated the study, obtained grants from public and private organisations, recruited the centres and is responsible for the follow-up of the cohort and for global analysis of data. She participated in performance of the research as a reference centre for Resistance Survey by resistance follow-up (genotyping, phenotyping and counselling) for the participating centres from the southern part of France, and was involved in routine virological follow-up, patient recruitment based on virological criteria and providing virological data, samples and isolates for the Limoges centre.

The French CMV Resistance Survey Study Group participants represent all the clinicians and the virologists that provided clinical and virological data from their centres and participated in patient recruitment.

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