Human enteroviruses and parechoviruses: disease spectrum and need for treatment in young children
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Chapter 7

Pleconaril revisited: clinical course of chronic enteroviral meningoencephalitis after treatment correlates with *in vitro* susceptibility

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Abstract

Background
Human enteroviruses (HEVs) can cause severe infections, especially in patients with a deficient humoral immune response, such as X-linked agammaglobulinemia. In this patient group, chronic enteroviral meningitis (CEMA) is feared because of extensive morbidity and high fatality rate. Treatment options consist of intravenous immunoglobulin (IVIG), with various outcomes. Pleconaril is an antiviral agent with *in vitro* activity against HEVs that has been used in the treatment of HEV infections.

Methods
The efficacy of pleconaril and IVIG against HEV isolated from the patients was assessed *in vitro* in two patients with CEMA.

Results
Echovirus 11 was found in the cerebrospinal fluid (CSF) of case 1. Treatment with high-dose IVIG and pleconaril did not provide any clinical improvement and HEV PCR in CSF remained positive. Case 2 (echovirus 13 positive in CSF) was also treated with IVIG and pleconaril. The patient recovered completely and HEV PCR in CSF became negative. Recent IVIG batches contained low titers of neutralizing antibodies against the patient strains. Echovirus 11 (case 1) was resistant to pleconaril *in vitro*, whereas echovirus 13 (case 2) was susceptible, in accordance with virological response after treatment and subsequent clinical results.

Conclusions
This is the first report that evaluates efficacy of antiviral treatment in CEMA patients in relation to *in vitro* susceptibility of clinical virus isolates. Since pleconaril is no longer available for compassionate use we strongly propagate that new drugs should be developed against these potential life threatening HEV infections.
Introduction

Human enteroviruses (HEVs) are single-stranded, positive-sense RNA viruses within the Enterovirus genus of the large Picornaviridae family. The genus Enterovirus contains >200 different serotypes known to infect humans, including polioviruses, echoviruses, Coxsackie A and B viruses, the numerically identified enteroviruses 68 to 109, and rhinoviruses. The immunological defence against HEVs including poliovirus greatly depends on the presence of neutralizing antibodies (nAbs). Therefore groups of patients with a deficient humoral immune response, like newborns and patients with primary or secondary immunodeficiencies are particularly susceptible to severe infections with HEVs. A severe and much feared condition in patients with primary immunodeficiencies, most particular in X-linked agammaglobulinemia (XLA), is chronic enteroviral meningoencephalitis (CEMA). XLA is a rare immunodeficiency due to an arrest in early B-cell differentiation, caused by mutations in the Bruton tyrosine kinase gene. This leads to severe hypogammaglobulinemia and markedly reduced B-cells. This condition makes patients with XLA particularly susceptible for disseminated or chronic HEV infections. The mortality in this group of patients is considerable with 35% of deaths due to disseminated HEV infections as was shown in a registry of patients with XLA in the United States.

Since the introduction and regular use of intravenous immunoglobulin (IVIG) in the treatment of humoral immune defects, the incidence of CEMA has decreased, although IVIG can not prevent all cases.

Treatment options for severe HEV infections are limited. In most patients treatment with IVIG is given, sometimes combined with intrathecal administration of immunoglobulins in the case of CEMA. Currently, no antiviral drugs are available against HEV infections. In the past, the antiviral compound pleconaril has been used on a compassionate-use basis in patients with severe HEV infections. Pleconaril blocks the binding of the enterovirus to cells by interfering with the capsid proteins of the virus and shows in vitro antiviral activity against most HEVs and rhinoviruses. The uptake and bioavailability of pleconaril is excellent, even in the cerebrospinal fluid (CSF). No serious adverse events were noted in treated patients. Currently, pleconaril is no longer available for compassionate use. However, the medical need for treatment in some patients with severe HEV infections can be urgent. We here describe two patients with CEMA that failed therapy with IVIG who were treated with pleconaril as a last resort. Our report is the first to correlate the clinical response in two CEMA patients with in vitro susceptibility testing of the infecting strains for pleconaril and IVIG. Our results will be put in perspective of previously published literature, further illustrating the need for antiviral treatment in severe HEV infections.
Methods

Virus detection, isolation and typing
HEV was detected by real-time reverse transcriptase PCR in CSF samples, stool samples, throat swabs, and EDTA blood samples from the patients as previously described.\(^\text{16}\) Virus isolation was performed by co-cultivation of PCR-positive CSF samples on different cell lines in 24-wells plates.\(^\text{17}\) Virus growth was observed by cytopathogenic effect (CPE). Culture isolates were characterized by serotyping with HEV-specific horse antisera pools A-G and H-R (RIVM, Bilthoven, The Netherlands).\(^\text{18}\) For genotyping of the culture isolates of the first patient, the nested approach as described by McWilliam Leitch et al.\(^\text{19}\) was used, while for genotyping of the culture isolates of the second patient, the seminested approach by Nix et al.\(^\text{20}\) was used, resulting in a 350-400 base pair sequence fragment of the VP1 gene. The VP1 sequence of the culture isolate was compared to VP1 sequences of HEV reference strains and phylogenetically characterized based on cluster analyses.

End point neutralization assay
Presence of nAbs in IVIG as well as serum obtained from case 2, was tested by neutralization assay. Only the IVIG that case 2 had received was available; case 2 had received two different batches of IVIG, including IVIG (batch 1: Sanquin, Amsterdam, the Netherlands) and Nanogam\(^\text{®}\) (batch 2: Sanquin, Amsterdam, the Netherlands). Serial 10-fold dilutions of virus isolates in concentrations ranging from 50% tissue culture infectious dose (TCID\(_{50}\)) of 10\(^\text{0}\)–10\(^\text{6}\) were incubated in 96-well plates with two-step dilutions of the IVIG batches or serum (undiluted, 1:5 - 1:20,480). Cell cultures were incubated for 7 days at 37\(°\)C and 5% CO\(_2\), and CPE was scored. Titres of nAbs were calculated by end-point neutralization.

Cytopathogenic effect inhibition assay
The 50% inhibitory concentration (IC\(_{50}\)) of pleconaril against echovirus 11 and 13 isolated from the 2 patients was calculated from a cell culture assay measuring inhibition of virus CPE by different concentrations of the compound.\(^\text{13,21}\) Coxsackievirus A9 (CAV9), Rhinovirus 16 (RV16; a kind gift of Koen F van der Sluijs, Laboratory of Medical Immunology, AMC, Amsterdam, the Netherlands) and human enterovirus 71 (HEV71) culture isolates were included as controls. Virus isolates in Eagle’s modified essential medium and 2% fetal calf serum were incubated at different concentrations (100, 200 and 500 TCID\(_{50}\)/50 μl) on 96-wells plates with a monolayer of cells (HT29 or Hel cells). Pleconaril was obtained from Sequoia Research Products Ltd (Pangbourne, UK). Pleconaril was tested in serial 10-fold dilutions (100-0.0001 μg/ml) in quadruplicate. Pleconaril was solubilized in dimethyl sulfoxide (DMSO) 40 mg/ml as described by Pevear et al.\(^\text{13}\), and pleconaril/DMSO solutions were prepared so that the DMSO end-concentration was the same in every well.\(^\text{13}\) Each plate included controls for virus growth, toxicity of DMSO on virus growth, toxicity of DMSO on cells, and cell growth. Cell cultures were incubated for 8 days at 37\(°\)C and 5% CO\(_2\), and CPE was scored. IC\(_{50}\) was calculated using the Reed and Muench\(^\text{22}\) method.
Results

Case 1 clinical course
The first patient was a man born in 1968, and in that same year XLA was diagnosed. This patient had a history of psychiatric problems starting at the age of 14, with episodes of neurological complaints. An enterovirus was cultured from the CSF in 1986, for which the patient received increased doses of IVIG selected for high titers against HEV. A liver biopsy in 2005 showed a chronic active hepatitis for which no cause was found.

In September 2007 the general condition of the patient was poor, with extreme fatigue and fever. The CSF showed 208 cells/mm³ and a total protein of 0.91 g/l. By PCR, HEV was detected in the CSF and in blood. The patient was treated with 25 g IVIG per week resulting in immunoglobulin (Ig) G levels in the blood of approximately 14 g/l. The clinical situation stabilized. In June 2008 an MRI scan showed quadriventricular hydrocephalus with transependymal oedema. In October 2008 treatment with pleconaril 400 mg three times daily for 10 days was started (Figure 1A). The therapy was well-tolerated, but there was no clinical improvement; 1 month after treatment, CSF PCR was still positive for HEV. The IVIG dose was increased to 30 g per week resulting in blood IgG levels around 18 g/l. Until October 2010 the condition of the patient remained the same without evident progression of neurological disease. In April 2010 he was admitted to the Psychiatry department for severe depression; 6 months later there was a sudden deterioration with a right-sided paresis, confusion, impaired consciousness and fever. After a few days, these symptoms disappeared. The CSF showed 132 cells/mm³, a total protein of 1.05 g/l and, again, a PCR positive for HEV. MRI showed no improvement compared to June 2008.

Case 2 clinical course
The second patient, a 12-year old boy with XLA for which he was treated with IVIG every 4 weeks, was doing well until September 2008. At that time he was admitted to the hospital because of headache, dizziness, nausea and vomiting, together with walking difficulties. He had no fever, C-reactive protein was <1 mg/l, and his levels of IgG, IgA and IgM were 6.0, <0.01 and <0.1 g/L, respectively. Neurologic examination showed ataxia and an MRI scan of the brain revealed a quadriventricular hydrocephalus and a diffuse enhanced signal of the meninges. The CSF showed a strongly elevated protein level (6.28 g/L), leukocytes of 170 cells/μL and red blood cells of 113.000 cells/μL. By PCR, HEV was detected in the CSF. Stool samples, EDTA blood and throat swabs remained negative for HEV. No other pathogenic microorganism was detected.

An Ommaya drain was placed because of the hydrocephalus. IVIG therapy was intensified to 18 g (0.5 gram/kg) every 3 weeks from the beginning of October, resulting in plasma levels of IgG of ≥9 gram/l. Because of persistent symptoms of ataxia, headache and nausea, treatment with pleconaril was started in November 2008 (Figure 1B). The drug was given orally at a dose of 600 mg per day (17 mg/kg/day), divided in three doses for 2 weeks.
The patient had no side effects of the antiviral compound, but the symptoms of nausea and ataxia initially did not improve. In December 2008, the boy was readmitted because of worsening of symptoms due to an increased hydrocephalus. A ventriculoperitoneal drain was inserted. At the same time, weekly IVIG from another batch (batch 2; Nanogam, Sanquin; 0.5 g/kg; Figure 1B) was started. The neurological symptoms improved rapidly and the patient was discharged from the hospital. In the following month, the nausea, headache and ataxia disappeared completely.

Figure 1. Effect of treatment with IVIG and pleconaril on PCR detection of enterovirus. Cycle threshold (Ct) value in relation to intravenous immunoglobulin (IVIG) and pleconaril administration in (A) case 1 and (B) case 2. A Ct value >40 is undetectable. CSF, cerebrospinal fluid; HEV, human enterovirus.
Virological response to treatment

Virological response measured by the cycle threshold (Ct) value of the real-time PCR is shown in Figure 1.

In case 1, the initial Ct value in CSF was 34.1, indicating low viral load (Figure 1A). After intensifying treatment with IVIG, the Ct value in the liquor seemed to increase, indicating a decrease in viral load, but a year later the viral load was around the same value as initially. Treatment with pleconaril in October 2008 did not give any change in CSF viral load and, since then, the viral load stayed in the same range despite further intensification of treatment with IVIG. The viral load in EDTA blood was initially low (Ct value 38) and became undetectable from 2008 except for one Ct-value of 38.9 in May 2008.

In case 2, the initial Ct value in CSF was 29.3, indicating moderate-low viral load (Figure 1B). Despite intensifying treatment with IVIG, the viral load in CSF remained in the same range. After start of treatment with pleconaril, the Ct value increased, indicating a decrease in viral load. After the pleconaril was stopped, initially the Ct value seemed to stabilize, but follow-up after readmittance and administration of Nanogam indicated a persistent decrease with subsequent undetectable viral load (Ct values >40) in January, which coincided with clinical improvement.

In vitro susceptibility for IVIG and pleconaril

In both cases, virus could be isolated from the CSF by cell culture. By serotyping, the culture isolates from case 1 and 2 could be identified as echovirus 11 and echovirus 13, respectively. Serotyping was confirmed by VP1-genotyping of the culture isolates.

Susceptibility of different virus strains to neutralization by the available IVIG batches was tested in vitro (Table 1). Both IVIG batches showed moderate to high nAb titres against CAV9, RV16, HEV71 and human parechovirus type 1 (HPeV1). However, very low nAb titres (<32) could be detected against echovirus 11 and 13 obtained from the patients. In addition, three sera from case 2 were tested for neutralizing capacity against echovirus 13 and HPeV1 after IVIG administration. None of the sera could neutralize the patient’s echovirus 13, while moderate to high nAb titres against HPeV1 could be found, in agreement with the level of nAbs titres in both IVIG batches (Table 1).

Susceptibility for pleconaril of the echovirus 11 and 13 isolated from the patients was tested by calculation of the IC_{50}. As positive controls, the viruses CAV9 and RV16 were tested and were found to be susceptible to pleconaril in vitro, with IC_{50} values in concordance with previous results;^{13,21} HEV71 was included as negative control and indeed was resistant against pleconaril.^{23} IC_{50} against pleconaril of both strains of echovirus 11 obtained from case 1 indicated that these strains were not susceptible. However, the IC_{50} of the strain of echovirus 13 obtained from case 2 was comparable to the susceptible control viruses (Table 1).
### Table 1. Susceptibility of enterovirus isolates for neutralization by IVIG and inhibition by pleconaril.

<table>
<thead>
<tr>
<th>Virus type</th>
<th>nAbs in IVIG&lt;sup&gt;a&lt;/sup&gt;</th>
<th>nAbs in serum of case 2&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Pleconaril IC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>μg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Batch 1 Batch 2 28-09-08 3-12-08 16-3-09</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Echovirus 13&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20 10 &lt;8 &lt;8 &lt;8</td>
<td>0.032</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Echovirus 11 (2007)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8 ND ND ND ND</td>
<td>&gt;32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Echovirus 11 (2010)&lt;sup&gt;d&lt;/sup&gt;</td>
<td>32 ND ND ND ND</td>
<td>&gt;32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAV9</td>
<td>1,280 1,280 ND ND ND</td>
<td>0.032</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RV16</td>
<td>320 160 ND ND ND</td>
<td>0.022</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HEV71</td>
<td>640 320 ND ND ND</td>
<td>&gt;100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPeV1</td>
<td>10,240 1,280 256 128 512 ND</td>
<td>ND</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Titre at a concentration of 100 50% tissue culture infective dose.  
<sup>b</sup>Echovirus 13 obtained from case 2.  
<sup>c</sup>Echovirus 11 obtained from case 1 in the year 2007.  
<sup>d</sup>Echovirus 11 obtained from case 1 in the year 2010.  
CAV9, Coxsackievirus A9; HEV71, human enterovirus 71; HPeV1, human parechovirus type 1; IC<sub>50</sub>, 50% inhibitory concentration; IVIG, intravenous immunoglobulin; nAbs, neutralizing antibodies; ND, not determined; RV16, Rhinovirus 16.

### Discussion

We here present the first report describing two cases of CEMA in which the efficacy of treatment with pleconaril and IVIG was verified *in vitro* on the virus isolated from the patients. We showed *in vitro* that recent IVIG batches contained very low nAb titers against echovirus 11 and 13 isolated from our patients, in accordance with absence of clinical improvement in both patients after intensifying treatment with IVIG.

We also showed that the echovirus 11 isolated from the first patient was resistant against pleconaril while the echovirus 13 from the second patient was sensitive to pleconaril in accordance with clinical observations.

Pleconaril has been used as treatment on a compassionate use basis in patients with immunodeficiencies and severe HEV infections. In a group of 17 patients with CEMA treated with pleconaril for 7-10 days, 75% showed a clinical response to therapy, while no serious adverse events were seen. In an adult case of chronic echovirus 13 meningoencephalitis, which persisted despite high doses of IVIG, the virus was cleared after treatment with pleconaril and the patient recovered, in agreement with our data. Further support for benefit of pleconaril in immunocompromised patients is anecdotic (Table 2), while cases with fatal outcome also have been described. In none of the severe HEV cases treated with pleconaril has its effect been properly evaluated; sometimes, HEV infections were not even proven, or the infecting serotype was unknown. Furthermore, pleconaril was almost always given in combination with IVIG, while the *in vitro* susceptibility of the infecting strains for IVIG and pleconaril was never tested in these clinical cases.

To our surprise, both echovirus 11 strains of case 1 were resistant to pleconaril, while laboratory strains of echovirus 11 were highly susceptible. Resistance could be due to adaptation of the virus and the forming of resistant strains in reaction to treatment with
pleconaril, but our patient was never treated with pleconaril or any other antiviral therapy before 2008. Several other family members had agammaglobulinemia, but none of them was ever treated with pleconaril. For CBV3, pleconaril resistance has been described in various laboratory and clinical strains due to a polymorphism in the hydrophobic pocket amino acid sequence. Between echovirus 11 strains, a large nucleotide variation can be found, dividing them in genogroups; therefore, it could be that some echovirus 11 strains carry polymorphisms leading to pleconaril resistance.

The role of IVIG in the treatment of patients with CEMA has not been clear. Variable results have been reported about the use of high-dose IVIG in CEMA, sometimes combined with intrathecal immunoglobulins, as reviewed by Misbah et al. and Crennan et al. In CEMA patients with echovirus 11 infection who were treated with IVIG, complete recovery as well as death have been reported. In one case, the echovirus 11 was neutralized in vitro with the IVIG batch the patient received, but in vivo the virus was still found in the CSF of the patient, despite high doses of IVIG and intrathecal immunoglobulin administration. This suggests that the in vivo concentration of nAbs in CSF was still not high enough to eliminate the virus.

Earlier studies on maternal antibodies indeed suggested that only high antibody titres correlate with protection against disease. We found low nAb titre against echovirus 11 in recent IVIG batches. Although the IVIG from case 1 was not available for testing, relatively low echovirus 11 nAb titres have been found in older IVIG batches as well, while echovirus 11 was one of the most frequently isolated HEV during that period. The genetic variation of circulating echovirus 11 strains could lead to antigenic differences in which nAbs against one genogroup do not neutralize other genogroups.

As shown for echovirus 11, the nAbs in the IVIG batches were low for echovirus 13 as well. In contrast to echovirus 11, isolation of echovirus 13 before the year 2000 was rare and it is considered an emerging cause of HEV meningitis in several countries during the past decade. Therefore, it could be that echovirus 13 nAbs are not yet highly prevalent in adult blood donors. Alternatively, as for echovirus 11, antigenic variation could prevent neutralization of specific genogroups by nAbs against other genogroups.

During clinical follow-up, the virological response of case 2 after administration of Nanogam suggested that this second IVIG batch had been effective in clearing the virus from the CSF; however, both first and second IVIG batches contained low echovirus 13 nAb titres and no echovirus 13 nAbs could be found in the blood of the patient, indicating that it must have been the pleconaril that cleared the virus.
### Table 2. Overview of case reports about treatment with pleconaril in patients with immunodeficiencies.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Age</th>
<th>Underlying disease</th>
<th>Symptoms</th>
<th>Virus (site of isolation)</th>
<th>Duration of pleconaril (time after initial symptoms)</th>
<th>IVIG given (time after initial symptoms)</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quartier et al.</td>
<td>9 Years</td>
<td>XLA</td>
<td>CEMA</td>
<td>Enterovirus (CSF)</td>
<td>7 Days</td>
<td>Prophylactic every 3 weeks, intensified during symptoms</td>
<td>CR (2 months after pleconaril)</td>
</tr>
<tr>
<td>Archimbaud et al.</td>
<td>14 Years</td>
<td>XLA, hepatitis</td>
<td>CEMA, hepatitis</td>
<td>Echovirus 11 (CF, blood, urine, stool)</td>
<td>7 Days (2nd month); 14 days (8th month)</td>
<td>Prophylactic, intensified during symptoms; Intraventricular Ig (11th month)</td>
<td>CR (from 16th month)</td>
</tr>
<tr>
<td>Nowak-Wegorzyn et al.</td>
<td>11 Months</td>
<td>SCID</td>
<td>Chronic diarrhoea, failure to thrive</td>
<td>Enterovirus (stool)</td>
<td>7 Days</td>
<td>No</td>
<td>Diarrhoea resolved (HEV-negative), died of haemolysis (unrelated to pleconaril)</td>
</tr>
<tr>
<td>Starlin et al.</td>
<td>9 Months</td>
<td>Omenn syndrome</td>
<td>Chronic diarrhoea, failure to thrive</td>
<td>Enterovirus (stool)</td>
<td>7 Days</td>
<td>No</td>
<td>CR of diarrhoea</td>
</tr>
<tr>
<td>Schmugge et al.</td>
<td>39 Years</td>
<td>Cystic fibrosis with renal and lung transplantation with immunosuppressive therapy</td>
<td>Acute flaccid paralysis, meningitis</td>
<td>Echovirus 19 (CF, blood)</td>
<td>10 Days (4th day)</td>
<td>Yes, for 5 days (4th day)</td>
<td>CSF negative for HEV, symptoms improved, died of respiratory failure after 2 months</td>
</tr>
<tr>
<td>Tormey et al.</td>
<td>26 Years</td>
<td>XLA</td>
<td>CEMA</td>
<td>Enterovirus (CSF)</td>
<td>10 Days (18h month)</td>
<td>Prophylactic every 4 weeks, intensified during symptoms</td>
<td>CR</td>
</tr>
<tr>
<td>Tormey et al.</td>
<td>18 Years</td>
<td>CVID</td>
<td>CEMA</td>
<td>Enterovirus (CSF)</td>
<td>10 Days (after diagnosis was made); 10 days (4th month)</td>
<td>Prophylactic every 3 weeks</td>
<td>CR (after 2nd course of pleconaril)</td>
</tr>
<tr>
<td>Schmugge et al.</td>
<td>44 Years</td>
<td>AIDS</td>
<td>Meningoencephalitis</td>
<td>Enterovirus (CSF)</td>
<td>10 Days (after diagnosis was made)</td>
<td>No</td>
<td>CR</td>
</tr>
<tr>
<td>Katsibardi et al.</td>
<td>11 Years</td>
<td>Non-Hodgkin lymphoma (in remission)</td>
<td>IAHS</td>
<td>Coxsackie B3 (blood, throat, urine, feces)</td>
<td>10 Days (after diagnosis was made)</td>
<td>Yes, single dose (after diagnosis was made)</td>
<td>Died of MOF (virus PCR-negative)</td>
</tr>
<tr>
<td>Cree et al.</td>
<td>33 Years</td>
<td>History of Henoch-Schönlein purpura, juvenile RA treated with immunosuppressive therapy and haemodialysis for IgA nephropathy</td>
<td>Meningoencephalitis, myocarditis</td>
<td>Coxsackie B4 (CSF, rectum)</td>
<td>10 Days (around 1 month)</td>
<td>No</td>
<td>Died at 53rd day after onset of symptoms</td>
</tr>
</tbody>
</table>

CEMA, chronic enteroviral meningoencephalitis in agammaglobulinemia; CR, complete recovery; CSF, cerebrospinal fluid; CVID, common variable immunodeficiency; HEV, human enterovirus; IAHS, infection-associated haemophagocytic syndrome; Ig, immunoglobulin; IVIG, intravenous immunoglobulin; MOF, multiorgan failure; RA, rheumatoid arthritis; SCID, severe combined immunodeficiency; XLA, X-linked agammaglobulinemia.
There are no precise data about the time needed to reach clinical and/or virological response for pleconaril, although in general viral clearance has been reported to occur within 2 weeks after treatment.\textsuperscript{24,26,28,29} It could be that our method of detection by real-time PCR is much more sensitive than previously used detection methods, resulting in a much longer time before viral clearance can be shown. In accordance with our results, Quartier \textit{et al.}\textsuperscript{25} suggested a delayed effect of pleconaril in two juvenile patients with CEMA who were treated with IVIG and pleconaril.

In 2002 the FDA rejected the use of oral pleconaril against the common cold.\textsuperscript{39} Currently, pleconaril is no longer available for use on compassionate basis in severe HEV infections. However, the great variety of clinical symptoms caused by different HEV strains in combination with insufficient follow-up of its antiviral effects may have contributed to earlier inconclusive results.

\textbf{Conclusion}

We here show that antiviral therapy against chronic enteroviral infections is necessary and can be clinically effective if the HEV strain is susceptible. Although this may seem obvious, clinical results with pleconaril in severe HEV infections have not been evaluated using our methods, and contradictory results may have been the results of a lack of \textit{in vitro} data. Therefore, more cases are needed to further determine the possibilities of \textit{in vitro} testing of response to antiviral treatment in patients with severe viral infections. We therefore strongly propagate that new drugs should be developed against these severe HEV infections, and that these drugs should be evaluated \textit{in vitro}, not only during development, but also during clinical evaluation, taking into account the variety of clinical syndromes as well as the biological and genetic variety of the circulating HEV strains.

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