Human enteroviruses and parechoviruses: disease spectrum and need for treatment in young children
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Chapter 13

Discussion
The first aim of this thesis was to describe the disease spectrum of human enterovirus (EV) (including human rhinovirus (HRV)) and human parechovirus (HPeV) infections in children, as these picornaviruses are one of the most prevalent pathogens causing disease in children. Knowledge on how to interpret the primarily technology-driven (molecular technology based) viral test results is lacking behind today’s technological possibilities with regards to detection of picornavirus infections in children. Therefore thorough insights in bridging laboratory results to clinical disease are needed. The second aim was to evaluate the need for treatment against these infections together with available treatment options and the role of neutralizing antibodies (nAbs) as possible effective treatment interventions. In this chapter the results of this thesis are put into perspective of the current knowledge.

**Rhinovirus C seems not to be associated with wheezing or more severe disease in the general population**

In Chapter 2 we showed that HRVs are the most prevalent viruses in young children with respiratory symptoms in an unselected birth cohort from the Netherlands. Although more severe infection was seen in the subgroup of HRV infected children without other viral co-infection, we did not find an association between wheezing or more severe disease and HRV-C infection. This is in contrast with an abundant number of recent studies, which showed that HRV-C is associated with more severe disease and asthma exacerbations. The majority of these studies were conducted in hospitalized patients suggesting that the disease spectrum differs between hospitalized patients (with severe disease) and the general population (mainly asymptomatic and mild illness). Indeed, other studies in birth cohorts and non-hospitalized symptomatic children did not show any difference in HRV-C related disease severity. Differences in host related factors and subsequently susceptibility for severe disease may contribute to this. Another possibility is that specific HRV-C types are more pathogenic than others and are overrepresented in hospitalized patients. Lee et al. found that the virulence of the HRV-B species in general was lower than the virulence of the HRV-A and -C species and that virulence varied between types. In agreement with our study no difference in wheezing or more severe disease between the HRV-C and HRV-A species was found in the birth cohort they studied.

The role of various HRV(-C) types and HRV(-C) induced host responses needs to be studied in order to clarify if variance in virulence of HRV(-C) types or host related factors or both play a role in the differences found between studies in hospitalized patient and population based cohorts. Therefore studies comparing HRV types between hospitalized patients, patients with mild disease and asymptomatic controls in the same period and region are needed.
Clinical relevance of HPeV infection

The clinical relevance of HPeV became evident since the discovery of HPeV3 and the subsequent association with sepsis-like illness (SLI) and meningoencephalitis in young infants. Together with EV, HPeV3 is the most frequent cause of aseptic meningitis in young children. The mere detection of these viruses by PCR in stool samples is insufficient for the determination of viral induced disease as these viruses are also detected in asymptomatic children and long-term (asymptomatic) shedding after symptomatic infection is frequently observed in children (chapter 4). We again confirmed our earlier findings on differences in disease association between HPeV1 and HPeV3 by comparing a larger group of HPeV infected children diagnosed by PCR in stool samples (chapter 3), showing that HPeV3 infected children were significantly younger and had more severe disease than HPeV1 infected children. Interestingly, low viral loads (high Ct-values) can be found in stool samples of HPeV3 infected infants with severe disease, suggesting that a positive HPeV3 is always clinically relevant. This is in sharp contrast to HPeV1 infection, which is mild or symptomless in most children. The clinical relevance of a positive HPeV1 PCR is part of an ongoing discussion. However, we showed that especially children with an underlying disease are indeed at risk to develop symptomatic HPeV1 disease (chapter 3). We also showed that HPeV1 is occasionally capable of causing severe disease (chapter 9), in agreement with previous reports. In addition, HPeV4, which up to now was associated with mild disease, might as well be able to occasionally cause more severe disease. This underlines the importance of HPeVs as clinical relevant viruses that should be incorporated in diagnostics on viral infection in children.

The long-term duration of shedding after symptomatic infection for several weeks to months is one of the reasons for the difficulties in interpretation of positive test results (in daily clinical practice). The gastrointestinal tract is the main site of replication for EVs as well as HPeVs, with the exception of HRVs, for which the respiratory tract is the main site of replication. It is possible that the detection of viral RNA by PCR in the gastrointestinal and respiratory tract is therefore merely representative of viral presence and does not always implicate invasive and therefore symptomatic disease. In addition, PCR does not discriminate between viable and non-viable viral presence. If the virus is found in otherwise sterile samples, for instance blood, CSF, myocard (in the case of myocarditis), this is considered a sign of invasive disease. Another difficulty in the interpretation of a positive stool sample is that stool samples can be incongruent, causing differences in viral load between different samples. However, in most children of our study viral load decreased slowly over time (chapter 4). The reason for long-term EV and HPeV shedding in feces in young children is not determined yet, but is possibly due to the immature immune system at young age. This long duration of shedding allows the virus to spread very efficiently among the population as young children often visit day care and have close contact with other children.
HPeV3; a disease of neonates?

Only very young children become severely ill from HPeV3 infection (chapter 3) and HPeV3 infection is hardly reported in older children and adults. HPeV1 infection is also mainly found in children below the age of 5 years. For HPeV1 this could be explained by the high seroprevalence in older children and adults. However, since HPeV3 seroprevalence is very low in children as well as in adults, other yet unidentified host or viral factors contribute to the development of HPeV3 disease in young infants. We found that mothers can be infected with HPeV3 (chapter 11), although they reported little or no symptoms in contrast to their children who experienced severe disease, suggesting a difference in disease susceptibility for HPeV3 between young children and adults. Another remarkable observation is that HPeV3 seems to behave different in other populations, such as reported for the Japanese population. Japanese seroprevalence is reported to be much higher than in the European population. Indeed, Japanese children are infected with HPeV3 at an older age (mean 12 months) and symptoms are usually mild, suggesting that in the Japanese population maternal nAbs protect against HPeV3 disease in the first months of life. In addition, an outbreak of epidemic myalgia associated with HPeV3 was reported recently in Japanese adults, showing that Japanese adults are susceptible for HPeV3 infection.

Although differences in circulating strains of HPeV3 can be responsible for variations in disease in different populations and patients, this is not very likely, since amino-acid similarity is very high (>99%) between contemporary circulating strains in Europe and the original Japanese strain. Host-related factors are probably more of influence in causing HPeV3 disease. Until now, little is known about the receptor usage of HPeV3. It could be that HPeV3 uses a specific receptor to disseminate throughout the body which is present in young infants but is down-regulated in older children and adults, explaining the absence of symptomatic disease at older age. The reason that the Japanese population seems to be more susceptible for symptomatic infection can be caused by differences in genetic host factors between populations. For EV71 it is shown that the class 1 HLA-A33 genotype is associated with an increased host susceptibility to EV71. This HLA type is found more frequently in the Asian populations than in Caucasian populations, providing a possible explanation for the high burden of EV71 related disease in Asia. Knowledge of the pathogenesis of HPeV3 infection and the (differences in) host responses is needed to prove if these hypotheses are true and is essential for the development of effective treatment options.

Need for treatment

Although most EV and HPeV infections are mild and self-limiting, these viruses are able to cause life-threatening infections as is reviewed in chapter 6. Life-threatening infections can be either due to specific viral serotypes or specific host factors. Examples are poliovirus...
and EV71 infections which can cause considerable neurologic morbidity and mortality. Although an effective vaccine against poliovirus is available since the 1950s, the virus is still not eradicated due to insufficient vaccine coverage and the emergence of vaccine-associated poliomyelitis. Anti-polioviral agents are therefore considered essential to obtain a polio-free world. These agents are needed in addition to vaccination strategies to treat new cases of acute poliomyelitis and eradicate persistent shedding in immunocompromised persons.\textsuperscript{12,13} In Asia the recent outbreaks of EV71 with high morbidity and mortality has led to an enormous scientific activity in search for vaccines and effective treatment. This has not been without success, since recently a promising vaccine against EV71 was developed in China.\textsuperscript{14} However, additional antiviral treatment against EV71 will probably still be needed in case of vaccine failure and for specific patient groups in whom vaccination is not indicated or effective (for example neonates and patients with primary immunodeficiencies), given the high morbidity and mortality rate due to brain stem encephalitis and acute flaccid paralysis.\textsuperscript{13,15} In the Western world research targeting antiviral treatment against EV and HPeV is lacking behind since the importance of EV and HPeV infections in these countries is not recognized. EVs and HPeVs only cause severe disease in a small subset of the population, making it less attractive for pharmaceutical companies to put a lot of money and effort in the development of antiviral drugs against these viruses. Most EV and HPeV infections are indeed benign and transient in young infants. However, this group is at risk for severe infections with permanent sequelae like meningoencephalitis, myocarditis, hepatitis and even infant death. Patients with primary immunodeficiencies are another patient category vulnerable for severe EV infections. In this thesis we present several cases of severe EV/HPeV infection, among others two patients with the severe condition of chronic enterovirus meningitis in agammaglobulinemia (CEMA). CEMA can cause significant morbidity and mortality in patients with agammaglobulinemia. Since the introduction of regular prophylaxis with intravenous immunoglobulins (IVIG), the risk of acquiring a chronic EV infection has lowered, but severe disease still occurs, particularly if IVIG does not contains sufficient protective nAb titers against the infecting EV type, as illustrated by our patients (chapter 7). Although this is a rare complication, the burden of disease is high in individual patients. The HRVs are another group of potential candidates for antiviral treatment. HRVs primarily cause the harmless ‘common cold’. However, the disease burden is high due to the high frequency of infection in the general population and the subsequent economical consequences (e.g. sick leave, visits to health care providers, and prescription of drugs). In addition, HRVs (and especially HRV-C) are recently recognized to also cause more severe disease like bronchiolitis, asthma exacerbations and lower airway infections. Similar to influenza, high infection rate and associations with severe disease makes the development of an anti-rhinoviral agent worth the effort for pharmaceutical companies. The lack of attention from biotech companies is also reflected by the low numbers of clinical trials with regard to anti-enteroviral drugs; six studies were conducted on anti-rhinoviral
drugs, one on anti-poliovirus drugs and two on anti-enteroviral drugs against severe EV infections. There is currently no antiviral medication against HPeV under development. Until now, none of the above mentioned clinical trials resulted in a successful registration of an anti-enteroviral drug. Of the many potential candidates, the capsid inhibitor pleconaril has been the most promising, although it was never licensed. In chapter 7 we described treatment with pleconaril as a last resort in 2 patients with CEMA and supported the clinical outcome with in vitro data on the susceptibility for pleconaril of the infecting EV strains. We showed that effectivity of pleconaril in vivo was consistent with in vitro susceptibility of the patient’s EV strain. Our results show that pleconaril is effective in life-threatening or chronic EV infections, but depends on the infecting EV type since not all EV types are susceptible for pleconaril. The earlier reported variances in outcome are probably the result of this difference in susceptibility between EV types since the infecting serotype was often not known or EV infection was not even proven. In vitro evaluation of susceptibility of different EV types can help to define those EV types against which pleconaril is effective.

The occurrence of resistance remains a considerable problem in both pleconaril treatment and drug development. We showed that pleconaril resistance can occur naturally and can be induced in susceptible strains in vitro (chapter 7 and 8). Ideally, the EV strain of the patient should therefore be tested for pleconaril susceptibility in vitro to predict the efficacy of treatment. The diversity of the Enterovirus genus together with the high mutation rate in most EV types make EVs extremely difficult to target effectively with one drug. In similarity with HIV, where combination therapy has been proven to be more effective in both adults and children, we and others suggest that a combination of at least two drugs, targeting different stages in the viral life cycle is eventually the best option for an effective anti-enteroviral treatment, minimalizing the risk of selecting resistant strains.

So far, pleconaril is the only anti-enteroviral drugs with proven efficacy and relative few side effects. In the absence of other treatment options (if IVIG treatment failed), withholding the use of pleconaril for patients with severe or chronic EV infections seems not ethical. The problem is that pleconaril is not available anymore, not even on compassionate use basis. Recently a clinical trial on the efficacy of pleconaril has been conducted in neonates with severe EV infections, but results are pending. Meanwhile registration of pleconaril on compassionate use basis could help to make this drug available for selected patients with severe or chronic EV infection.

Although pleconaril seems the most promising candidate to use in severe EV and HRV infections, it is not effective against today’s most life-threatening EVs such as EV71 and poliovirus. Many drugs with a variability of potential targets are currently under investigation because of their presumed activity against EV71 but none of these drugs are evaluated in clinical trials. For the treatment of poliovirus, the capsid inhibitor V-073 seems the most potential candidate in vitro. In addition, the HPeVs, including HPeV3, are not susceptible for pleconaril. No antiviral drugs against HPeV are currently under development. However, since HPeV3 is causing severe disease in neonates and young infants, and little effect of
IVIG is expected because of the lack of specific nAbs, effective treatment for this particular patient group is urgently needed. Therefore HPeVs should be included in the ongoing research for anti-enteroviral drugs.

A relative new approach in search for an effective treatment is to target host cell factors that are essential for viral replication. This strategy might have two major advantages; broad-spectrum activity and a reduced likelihood of the development of resistance. Promising candidates are inhibitors of the host cell factors Hsp90 and phosphatidylinositol 4-kinase III (PI4KIII). These inhibitors indeed show a broad spectrum of anti-enteroviral and antirhinoviral activity.22,23 But a major drawback is the occurrence of resistance against PI4KIII inhibitors,24 making these agents unsuitable for use as monotherapy.

Antibodies as treatment option

We used HPeV infection in young children as a model to prove or reject the hypothesis that (maternal) nAbs are of importance in EV and HPeV infections (chapter 11). In a case-control study we showed that there were no protective HPeV3 nAbs in mothers of neither cases nor controls. In contrast, HPeV1 nAbs were seen in 99% of the mothers of both cases and controls. Although no differences in seroprevalence were seen between case and control mothers, we found that HPeV3 infected children were mainly under the age of 3 months, while children with HPeV1 infection were significantly older. These results suggest that (maternal) HPeV1 nAbs are important in protection against (severe) disease (because HPeV1 infection is virtually absent in children <3 months) and therefore could serve as a candidate for treatment. Illustratively, treatment with IVIG in an infant with severe HPeV1 associated dilated cardiomyopathy led to a substantial rise in nAb titers and clinical improvement (chapter 9). The used batch of IVIG contained high titers of aHPeV1 nAbs, in line with the observed improvement. IVIG contains immunoglobulins of more than thousand donors and is a good reflexion of the circulating EV and HPeV types in a specific population. But due to variations in circulating EVs and HPeVs between populations and over years, nAb titers vary per batch, hampering an accurate prediction of effect of treatment with IVIG. This could explain the differences in outcomes of critically ill EV infected patients after treatment with IVIG. For example, Chinese adult donors showed to have high titers of EV71 specific antibodies, providing a rationale for treatment with IVIG in case of severe EV71 infections. Retrospective studies indeed demonstrated a beneficial effect of IVIG when given early in the course of EV71 infections and several countries incorporated the use of IVIG in their treatment guidelines.25 IVIG treatment of children with severe HPeV3 infections is not likely to be effective, since antibody titers are very low in the general population in Europe and thus in IVIG products.4 A more targeted approach would therefore be the development of monoclonal antibodies.
A remarkable outcome was that virtually none of the mothers had nAbs against HPeV3, while this virus is circulating for at least 20 years, suggesting a different mechanism of infection and protection. Even after a proven HPeV3 infection, no or only partial inhibition of HPeV3 was found in vitro in 2 mother-child pairs (own observations), suggesting that other immune responses are of importance in the defense against HPeV3 infection and high nAb titers are not needed to clear the virus. The absence of production of nAbs after HPeV3 infection in mothers can also be due to the fact that the gastrointestinal tract is only colonized with HPeV3 and does not elicit infection by becoming invasive (because specific receptors are missing) and therefore no production of nAbs is needed. However, in children a sustained or temporary rise in aHPeV3 nAbs was seen in some cases, suggesting that nAbs might play a role as well. More research is needed to elucidate the role of nAbs in the host response against HPeV3 infection.

The innate immune system; mistakenly overlooked?

Toll-like receptors (TLRs) are involved in the immune response against EVs, HRVs and HPeVs. TLR8 is thought to play a role in the pathogenesis of white matter injury in HPeV3 and EV central nervous system (CNS) infection in young children. Experiments in mice showed that TLR8 is also localized in neurons and axons of the developing brain and is responsible for the regulation of axonal growth and development only in the perinatal period. Activation of TLR8 leads to release of reactive oxygen, nitrogen species and pro-inflammatory cytokines which are toxic for neural cells. This could explain why HPeV3 and EV related encephalitis is mainly seen in young infants. TLR8 is also involved in immune-responses in patients with Coxsackie virus B (CV-B) induced dilated cardiomyopathy; increased TLR8 levels were related to adverse clinical outcome. Polymorphisms in TLRs are frequently seen and are considered of importance in the individual susceptibility for infection caused by microorganisms. For example, genetic variations in TLR3 change the host susceptibility for CV-B3 myocarditis. In addition, polymorphisms of promoter regions of cytokines can influence the cytokine release and subsequently the severity of symptoms. Polymorphisms in the promoter region of IL-6 and IL-10 seem of importance in symptom severity of HRV infections and probably influence the susceptibility for development of asthma. Studies in China showed that polymorphisms in IL-6, IL-10, IL-17 and IFN-γ play a role in the development of encephalitis in EV71 infected patients. These polymorphisms in TLRs and cytokines could explain differences in susceptibility and disease spectrum between individuals and even whole populations. Future research focusing on host responses against EV and HPeV infections is needed to clarify the role of polymorphisms in pathogenesis of disease between different populations.
Conclusions

HPEVs and EVs are amongst the most prevalent disease-causing viruses in children and elicit a wide range of disease from mild illness to life-threatening infections. Treatment is necessary and urgently needed in severe EV and HPEV infections. NAbs play an important role in the host defense against HPEV and EV viruses, but cannot fully explain the differences in pathogenesis of disease of various serotypes of HPEV and EV. Differences in receptor usage, viral virulence factors and host related responses of the cellular and innate immune system are largely unknown but probably play an important role in host defense. Knowledge of the pathogenesis and host response against EVs and HPEVs is essential to develop effective treatment strategies.

Meanwhile treatment with IVIG, ideally selected for its high nAb titer against the infective EV or HPEV type, is the most feasible option in life-threatening EV and HPEV infections, awaiting the development of monoclonal nAbs. The development of effective antiviral drugs should start with the registration of pleconaril (on compassionate use basis), making this broad spectrum anti-enteroviral drug available for those patients with severe EV infections in whom antibody-based therapy has failed or is likely to fail. The search for other effective drugs, targeting the different aspects of the life cycle of the virus and/or host-related factors should meanwhile be intensified.
References


