General discussion
INTRODUCTION

This thesis consists of studies on Enterovirus (EV) and Human Parechovirus (HPeV) infections in children and focuses on the clinical symptoms, diagnostics and outcome of disease. Interactions between the pathogen and host determine the severity of EV and HPeV infections. For a better understanding of these pathogen and host interactions, it is important to understand the pathogenesis of EV and HPeV infections. Though the studies in this thesis did not primary focus on the pathogenesis of these viruses, I will limit myself here with giving a short overview of the pathogenesis of their infections.

The pathogens. It is known that EV and HPeV are among the most frequent causes of viral infections globally.14 The exact prevalence and incidence of their infections are unknown, since they are not notifiable infections. Ninety percent of children have been infected with EV or HPeV by the age of two years.1,5,6 EV and HPeV are more often detected as cause of illness since the introduction of molecular diagnostics. Because each year new types of HPeV are discovered, it is expected that the incidence rate will become higher in the coming few years. Children can present themselves with different symptoms, partly dependent on the genotype of the virus and different host factors.

The host. It is mostly young children, who are infected with EV and HPeV. It is interesting to see that approximately 40% of these young children present with a meningitis, without pleocytosis. Why do some children and not others have meningitis without pleocytosis? I will try to explain this hereunder. The difference in host severity of disease, may be caused by differences in immune responses, genetic variation or both. This is elaborated further below. All children in the cohorts included in this thesis were not so severely ill to require hospitalization in an intensive care unit. This contrasts with papers on Asian children infected with EV-71, who had a high prevalence of morbidity and mortality.7 Reasons for the differences in clinical presentation and severity are presented in this discussion chapter. As with severity, the outcome of EV and HPeV infection depends on the infecting organism, on the one hand and the host factors, such as location of infection, on the other hand. It is known that bacterial meningitis in children are associated with neurological sequelae.8,9 There are also reports of impaired neurological outcome in Asian children after an EV-71 meningo-encephalitis.7,10 There is not much known about the prognosis of EV and HPeV infections in non-Asian children. In this chapter, I will discuss the different possible mechanisms involved in the neurological outcome after EV or HPeV meningitis. Finally, I will make proposals for future research perspectives.
OVERVIEW OF THE PATHOGENESIS OF EV AND HPEV INFECTION

Transmission of EVs and HPeVs can occur through different routes, including the fecal-oral, transplacental, respiratory droplets or vesicular fluid.\textsuperscript{11,12} The primary replication sites of EV and HPeV are the epithelial cells of the oropharyngeal and gastrointestinal mucosa. There, they bind to specific receptors on enterocytes and cross the intestinal lining cells, perhaps with replication but without apparent cytopathicity. Significant virus replication takes place in the Peyer’s patches of the lamina propria.\textsuperscript{13} This is followed by a minor viremia, which may lead to a secondary site of tissue infection in multiple organ systems such as the central nervous system (CNS), heart, lungs, liver, muscles and skin. When virus replication is controlled by host defense mechanisms, children experience asymptomatic infection. When this is not controlled, more significant replication at these other sites results in a major viremia associated with the signs and symptoms of viral infection. If the CNS has not been seeded during the initial minor viremia, this may occur during the major viremia.

Pathogenesis of CNS infection

There are two main routes of entry of EV and HPeV into the CNS. The first one is via the blood supply. Once inside secondary lymphoid tissues, virus particles are often shed into the blood stream, resulting in systemic infection.\textsuperscript{14,15} The CNS parenchyma is protected from harmful substances in the blood by the blood brain barrier (BBB), which is formed by brain microvascular endothelial cells.\textsuperscript{14,16} However, viruses have adapted more ways to overcome this obstacle.\textsuperscript{15} The virus may directly infect vascular endothelial cells, which allow direct passage across the BBB into the CNS. Additionally, there are areas of the CNS such as the choroid plexus and circumventricular organs that are not completely protected by the BBB and serve as entry points for several viruses. Infected hematopoietic cells are also used as “Trojan horses” to transport the virus into the CNS by crossing the BBB via the blood supply. Finally, systemic viral infections can lead to inflammation-induced breakdown of the BBB, allowing viruses to slip through the cracks into the CNS.\textsuperscript{14} This concept of viremic spread to CNS is also supported by some studies.\textsuperscript{17,18} As a second major route of CNS entry, EVs and probably also HPeVs, may infect and migrate through peripheral motor neurons. By binding to specific receptors of neurons at the neuromuscular junction, they can spread into the neuronal cell body by the fast axonal retrograde transport system.\textsuperscript{15} Via somatic motor nerves, they infect anterior horn cells and then migrate up to the corticospinal tract to the neurons in the motor cortex.\textsuperscript{19} Once the viruses reach the CNS, viral tropism and the ensuing
immune response combine to shape the resulting disease. Viruses that remain within cells of the meninges induce meningitis, whereas those that infect the CNS parenchyma cause meningoencephalitis, encephalitis, or myelitis. The cells of the leptomeninges are renewable but neurons, which are long-lived, terminally differentiated cells, are not.\textsuperscript{20}

**Immune response to viral CNS infection**

The normal, uninfected CNS lacks immunological activity. Following a CNS infection, fast protective responses occur.\textsuperscript{20} EVs and HPeVs are recognized by toll-like receptors (TLRs) and retinoic acid-inducible gene I-like receptors.\textsuperscript{21,22} Interferon-β (IFN-β) slows virus spreading and constrains virus replication before induction of virus-specific immune responses, chemokines and pro-inflammatory cytokines are produced. It results in further activation of microglia and increases expression of adhesion molecules by endothelial cells. Particularly, mononuclear inflammatory cells infiltrate the CNS and initially accumulate in perivascular areas. From there they infiltrate the parenchyma in the regions of virus infection. Essentially all components of the cellular immune response are detected in the CNS during EV and HPeV infections. Natural killer cells are detected first, followed by antigen-specific CD4\textsuperscript{+} and CD8\textsuperscript{+} T-cells, then B-cells and monocyte/macrophages. T-cells produce additional cytokines, such as IFN-γ, interleukin-6 (IL-6) and IL-10, and the B-cells produce antibody, mainly of the immunoglobulin IgG subclasses, which initiates the clearance of virus from infected cells. The virus is cleared from cells in the brain parenchyma by inhibition of virus spread to new cells and elimination of cell-free infectious virus.\textsuperscript{20}

**REASONS FOR DIFFERENCES IN CLINICAL PRESENTATION AND SEVERITY OF EV AND HPEV INFECTIONS**

EV and HPeV both belong to the family of the *Picornaviridae* and have similarities in their manifestations. EV and HPeV infected children can present with different clinical symptoms, as described in chapter 2 and 3. These include fever, malaise, feeding problems, vomiting, nausea, abdominal pain, diarrhea, coughing, rhinitis, dyspnea, irritability, drowsiness, nuchal rigidity, photophobia, sore throat, earache, aphtha, conjunctivitis, rash, and/or paralysis.\textsuperscript{23-28} However, as the clinical spectrum of these infections is diverse and nonspecific, it is difficult to recognize an EV and HPeV infection only on the basis of symptoms. Verboon-Maciolek et al. compared the clinical signs of 21 EV infected and 11 HPeV infected neonates.\textsuperscript{29} They could not distinguish neonatal EV from HPeV infection on the basis of clinical signs.
Contrastingly, we found that EV infection was more associated with meningitis and HPeV infection more with gastro-enteritis. A possible explanation for this difference may be the difference in study populations. Verboon-Maciolek and colleagues only included neonates (some of whom were preterm births) in a neonatal intensive care unit. We included children from 0–16 years old, who presented in three general hospitals. Furthermore, we included more EV positive children than Verboon-Maciolek et al. A final explanation could be the predominance of some genotypes in our study, since different EV and HPeV genotypes can give different symptoms depending on which organ system is mostly infected. The HPeV genotypes were the same in both studies, but the EV genotypes were different. In chapter 3 of this thesis, Echovirus 30 was the most frequently detected EV genotype (n=18), while in the study of Verboon-Maciolek et al. none of the children were infected with Echovirus 30 and their most frequent detected type was coxsackievirus B2-B5 (n=7). Some EV genotypes tend to be more severe, for example EV-71 and EV-D68. Recently there were outbreaks with EV-71 (1997) and EV-D68 (2014.) The outbreak of EV-71 across the Asia-Pacific region was associated with hand, foot, and mouth disease and severe neurological complications. EV-D68 caused severe respiratory disease, with considerable morbidity, requiring intensive care hospitalization and mortality among children in the United States. In the studies presented in this thesis, we found only one child infected with an EV-71 genotype. In none of the children did we detect an EV-D68. The low prevalence of both genotypes in our study population is comparable to the data of the National Institute for Public Health and the Environment (RIVM).

Why does the same EV or HPeV genotype cause different clinical spectra and do some genotypes cause more severe infections than others? A possible explanation for the difference in severity of infection between genotypes are related to host factors, such as age of the infected child. Infection at a younger age seems to cause a more severe illness than at an older age. This, is supported by the results of this thesis, presented in chapters 2 and 3. This observation is further supported by a report of HPeV-3 infection in both mothers and their young children. While the mothers in that study reported little or no symptoms, their children experienced severe disease, suggesting a difference in age-related disease susceptibility for HPeV-3. This may be explained by a presumably less cross-protective immunity from previous outbreaks, by younger children. Young age is therefore a risk factor for severe EV or HPeV disease. There may also be a lack of maternal protection or failure of antibody protection, in addition to other host and/or viral factors involved in susceptibility to these viruses. Another hypothesis that could explain the severity of infection is the role of
coinfection. We were unable to confirm this hypothesis, as patients with a coinfection in our study population were excluded from the analyses in this thesis. Differences in circulating strains of EV and HPeV genotypes have been suggested to be responsible for variations in clinical severity of disease in patients. This is not very likely, since amino-acid similarity is very high between circulating EV and HPeV strains (for example >99% for HPeV3). Some have also suggested differences in the biological characteristics of the viruses, such as cell tropism. HpeV-3 is more difficult to grow in cell culture than HPeV 1. HPeV-3 lacks the arginine-glycine-aspartic acid (RGD) motif located in the C terminus of the capsid protein VP1, which has been shown to be essential for HPeV-1 receptor binding and entry. The differences in in-vitro growth characteristics and the lack of the RGD motif imply use of a different cellular receptor by HPeV-3 from that used by HPeV-1, and a potentially different cell tropism.

Host-related factors may be associated with a higher variability in clinical severity. Perhaps the receptor usage for EV and HPeV is down regulated in older children and adults, since most of the infected patients are younger children. This might explain why older children and adults have less severe symptoms of infection. Finally, differences in host genetic factors between individuals and populations could explain the variability in clinical severity. It is shown that the class 1 human leukocyte antigen (HLA) A33 genotype is associated with an increased host susceptibility to EV-71. This HLA type is found more frequently in the Asian than in Caucasian populations, providing a possible explanation for more serious EV-71 related disease in Asia. Polymorphisms of TLRs could also be involved in individual susceptibility for infection. For example, genetic variations in TLR-3 changes the host susceptibility for Coxsackievirus-B3 myocarditis. Polymorphisms of promotor regions of cytokines can influence the cytokine release and the severity of symptoms. Since the studies presented in this thesis did not focus on the pathogenesis of EV and HPeV infection, the above mentioned hypothesis could not be confirmed.

**MENINGITIS WITHOUT PLEOCYTOSIS**

In chapter 2 we describe a retrospective study on children with EV meningitis without pleocytosis. We found that these children were significantly younger, experienced more drowsiness, had lower white blood cell (WBC) counts and higher C-reactive protein (CRP) levels, compared to those with pleocytosis. This observation was supported by the findings in our prospective cohort study (chapter 3). This observation is relevant, because
The detection of CSF pleocytosis is an important biochemical marker of meningitis in the general pediatric clinical practice.

The mechanism underlying the lack of pleocytosis in children during EV or HPeV meningitis is still unclear. A first possible explanation could be that the children present in an earlier stage of illness, in which the production of (pro) inflammatory cytokines has been stimulated, but the blood leukocytes have not yet infiltrated the cerebrospinal cavity.\textsuperscript{55} Nevertheless, we found that patients with EV meningitis without pleocytosis did not have a shorter duration of symptoms than those with pleocytosis. This is comparable to other studies, although a significant relation has been reported between an early lumbar puncture, within 48h of symptoms, and absence of pleocytosis.\textsuperscript{56} In our prospective cohort study, we found that children with HPeV meningitis without pleocytosis had lower CRP levels compared to those with pleocytosis. These observations suggest that there might be other explanations. Some children have higher viral loads than others; it is possible that only those with high viral loads in their blood and/or CSF develop pleocytosis. This is supported by the findings of Volle et al. that viral loads were higher in the presence of pleocytosis in comparison with the absence of pleocytosis and in neonates compared with infants and children.\textsuperscript{57} The association between higher viral loads and the presence of pleocytosis was only observed in adults and newborns but not in children and infants, which is most probably explained by a small sample size. Another explanation for EV/HPeV meningitis without pleocytosis could be the age-related immaturity of the specific immunity against EV and HPeV. In support of other studies, we found that children with EV and HPeV meningitis without pleocytosis were younger than those with pleocytosis.\textsuperscript{56-59} Possibly, the chemokine response required for the recruitment of leukocytes to sites of infection was not yet ripe enough in young patients\textsuperscript{58} and peripheral WBC can be identified as a factor, which independently influences CSF pleocytosis.\textsuperscript{60} Further prospective and experimental studies are needed to clarify the temporal age-related relationship and mechanisms involved. A final hypothesis is that the absence of pleocytosis could be related to genotypic features, which is supported by the observation that the CSF viral loads of patients with Echovirus 30 meningitis were higher than in those with Echovirus 6 meningitis.\textsuperscript{57} For pediatricians it is important to know that EV or HPeV meningitis cannot be excluded only on the bases of the absence of pleocytosis. Therefore, we are of the opinion that detection of genomic RNA with RT-qPCR has an additional value in the diagnosis of EV and HPeV meningitis and must be performed in each child who presents with symptoms and signs of EV and HPeV meningitis.
DIFFERENT MECHANISMS FOR POSSIBLE NEUROLOGICAL SEQUELA AFTER EV OR HPEV MENINGITIS

Not much is known about the outcome of EV and HPeV infections in non-Asian children in Western countries. Contrasting results are described in small studies that have been performed in different study populations. To our knowledge, the studies in this thesis are the first to prospectively describe the neurodevelopmental outcome of a large cohort of children with EV and HPeV infections in children in a Western country. As described in chapter 6, we found that the motor development of children two years after an EV or HPeV infection is not impaired, compared to those without infection. In chapter 7, we describe the cognitive functioning, as well as behavioral problems in children after an EV and HPeV infection. These were not impaired between children with EV and HPeV meningitis, compared to both children with EV and HPeV infection elsewhere in the body and those in whom no pathogen was detected, after two-years of follow-up. This is an important finding, since EV and HPeV are frequent causes of infection in childhood. With the study results presented in this thesis, parents and children can be better informed about the most likely disease outcome.

Both EV and HPeV are neurotropic viruses, known to cause CNS infection and may lead to neurodevelopmental delay in children, but the severity may be different between different genotypes. Studies have reported impaired neurodevelopmental outcome in children with an EV-71 meningitis or meningoencephalitis,61-63 but not much is known about the outcome after other EV or HPeV genotypes. EV-71 is not as prevalent as other EV types in the Netherlands. EV-71 mostly infects neurons which are not renewable. This makes the chance of neurological sequela higher than infection with EV or HPeV genotypes, which do not infect neurons.64 The severity of the acute illness and associated acute neurologic complications might correlate with sequelae. This might explain why we did not find any significant neurodevelopment delay between the 3 study groups of children. As shown in experimental studies, the dose and route of virus transmission can markedly influence the outcome of a virus infection. Minimal doses may be subclinically controlled by the innate defense mechanisms and may be insufficient to induce adaptive immune responses. Massive doses can overwhelm these immunological defense mechanisms and cause severe disease, for example via direct cytotoxic effects of viral components. In our studies, we did not determine the viral load of EV and HPeV infection. Instead we used the PCR cycle threshold values of the EV and HPeV positive patients in the different body specimens, which is a semi-quantitative measurement of the viral load. Further analysis of these data can be used to support this hypothesis. Another hypothesis for an impaired outcome could be that there is a coinfection,
but those children were excluded in the studies in this thesis. Coinfections with EV-71 have been reported, but there was no evidence of an increased likelihood of CNS disease in such patients, which makes this hypothesis less likely to be applicable to our study population.\(^6^5\)

The outcome depends on the tissue damage caused by the immune reaction, host factors that limit tissue disease and factors that favor tissue damage.\(^6^6\) The tissue damage caused by the immune system depends on the contributions of both the innate and adaptive immune responses. The pattern of innate immune events induced after the entry of the virus may dictate the outcome of infection. Adaptive immune effectors, such as cytokines and IFNs, can contribute to tissue damage. T-cells for example, can directly destroy virus infected cells or release cytokines such as tumour necrosis factor, which damages the cells. Furthermore, antibody responses to viruses may also contribute to tissue damage, through complement activation resulting in an inflammatory reaction. It is known from bacterial meningitis that the greater the host's inflammatory responses to the microorganism and its products, in the subarachnoid space, the greater the likelihood of permanent sequelae.\(^6^7\) There are several responses which the host can make to minimize tissue damage. These countermeasures include 1) the production of cytokines such as IL-10 with an anti-inflammatory activity, 2) blockage of pro-inflammatory cytokine and chemokine production and 3) interference with the signaling of several pathways, which lead to pro-inflammatory cytokine production. The extent of IL-10 induction during an infection could influence the amount of tissue damage that occurs. For instance, if the IL-10 response is lacking, owing to genetic mutation, or is artificially suppressed by antibodies to IL-10 or its receptor, inflammatory reactions to infectious agents may become exaggerated.\(^6^6,6^8\) Moreover, several other natural host products can participate in the control and resolution of inflammatory reactions, for example galectin. Galectin-9 binds to the T-cell immunoglobulin domain on activated effector cells and causes apoptosis, but at the same time it expands the regulatory T-cell response, minimizing tissue damage.\(^6^6,6^9\) Another mechanism of counteracting excessive tissue damage is the induction, activation or expansion of several types of regulatory T-cells, which can inhibit the function of other cell types. Whether a virus causes severe tissue damage also depends on the age at which infection occurs. Increased susceptibility of the young, especially neonates, has been attributed to immature responsiveness of the immune system, particularly components of innate immunity.\(^7^0\) In the immature brain, cytokines and perivascular accumulated lymphocytes may lead to white matter damage.\(^6^4,7^1\) Further prospective studies are needed to test these interesting hypotheses, since our studies were not designed for this purpose.
FUTURE PERSPECTIVES

This thesis has provided more insight into the clinical, diagnostic and prognostic spectrum of EV and HPeV infections in children, but many questions still remain. The ideal setting to investigate EV and HPeV infections in children would be a national collaboration by the setup of a working group. Since EV and HPeV are frequent causes of infection in children, it would be good if a national collaboration can be started. For example, the department Medical Microbiology of the Academic Medical Centre Amsterdam is an internationally known expert centre of HPeV infection, the RIVM is an international leading expert in genotyping and vaccine development for EV infection and the Neonatology Department of the Wilhelmina Children's Hospital in Utrecht has published extensively on neonatal EV and HPeV infections. Besides these bigger institutions, there are also other general hospitals interested in EV and HPeV infections. It would be a great opportunity to combine all the knowledge of these institutions to create a national online database for EV and HPeV infections, in which for example epidemiological, clinical, diagnostic, microbiological, and prognostic information can be collected for further research.

Because the clinical spectrum and outcome of EV and HPeV infections are diverse, it would be interesting to make clinical decision rules which can predict the neurodevelopmental outcome of different genotypes of EV and HPeV. For example, the gender and age of the patient, laboratory results of the blood (CRP, WBC) and CSF (WBC, protein) and EV and HPeV genotypes can be included in those rules. To my knowledge, there are no clinical decision rules yet for this purpose, but the introduction of a national network could be the first step in making those.

Regarding the diagnosis of EV and HPeV infections, it is important to keep validating and updating the existing EV and HPeV assays, since the number of novel HPeVs identified over the last few years has increased considerably and probably will continue to increase further in the next years.

It would be better if we could prevent EV and HPeV infections. Until recently, there were only three vaccines available for infections with Picornaviruses, namely poliovirus, hepatitis A virus and veterinary foot-and-mouth disease. After the recent outbreaks of EV-71 in Asia, with both high morbidity and mortality, new vaccines against EV-71 have been developed. Although the results look promising and the vaccine has just recently become available in the private market in China, it is not expected that vaccination against EV-71 will be introduced in Europe any time soon. Large outbreaks, with high morbidity,
have not yet been observed in Europe and the genotypes causing these outbreaks in Asia circulate only to a low extent in Europe. However, this could change in the future and the experience with EV-71 vaccination, which is now being gained in China might be valuable. When more vaccines for EV and HPeV infections are developed, they will probably not be included in the national vaccination program. Firstly, the genotypes known to cause poor clinical and neurocognitive outcomes (such as EV-71 and D68) are not very prevalent in the Netherlands, although an upsurge of EV-D68 in the Netherlands is recently described. Moreover, the genotype used in the Asian vaccines (genotype EV-71 subtype C4), responsible for the outbreaks in Asia, is rarely seen in Europe (in Netherlands there is a circulation of EV-71 subtype C1 and C2). Furthermore, it is probably not worth it to develop vaccines for the other EV and HPeV genotypes, since they cause less severe infections. An important difficulty in the development of vaccines is that there are a lot of different serotypes. In addition, it is not clear at the moment what the cost-effectiveness of an EV or HPeV vaccine will be in our country. Though younger children are more vulnerable to severe infections and have a higher risk of an impaired outcome, such an inactivated whole-virus EV or HPeV vaccine, can be given only from the age of 6 months. Unfortunately younger children, who constitute the target group will not benefit from the vaccine but from herd immunity. Moreover, additional antiviral treatment will probably still be needed in case of vaccine failure and for specific patient groups in whom vaccination is contra-indicated or ineffective (such as neonates and patients with primary immunodeficiencies). To date no antiviral drugs have been approved for the treatment of picornavirus infections. Since EV and HPeV infections only cause severe disease in a small subset of the population in Western countries, it is less attractive for pharmaceutical companies to invest a lot of money and effort in the development of new antiviral drugs.

**MAIN CONCLUSIONS OF THIS THESIS**

1. In this study population we found that EV infection was more often associated with CNS infection, such as meningitis whereas HPeV infection was more often associated with gastrointestinal infection. The clinical symptoms of EV and HPeV infections varied.

2. Molecular diagnostics (reverse real-time quantitative polymerase chain reaction (RT-qPCR) or GeneXpert) are superior in comparison to viral culture in detecting EVs and HPeVs. RT-qPCR is superior in comparison to GeneXpert in detecting EVs.
3. Molecular diagnosis of the CSF is needed to diagnose EV and HPeV meningitis, since this cannot simply be excluded by the absence of CSF pleocytosis.

4. RT-qPCR of the feces has the highest sensitivity for EV and HPeV RNA detection in symptomatic children. CSF and blood samples are also useful for detecting EVs and HPeVs RNA. Nasopharyngeal or urine samples appeared to be less useful.

5. Impaired motor development of children 24 months after an EV or HPeV infection was not observed. We did not find any difference in cognitive development of children with EV or HPeV meningitis compared to those with an infection elsewhere or those in whom no pathogen was detected. However, older age at diagnosis was associated with lower verbal intelligence quotient and behavioral problems, especially in children with EV infection.

REFERENCES


