Advances in congenital and postnatal cytomegalovirus infections
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Advances in congenital and postnatal cytomegalovirus infections

Ontwikkelingen in congenitale en
postnatale cytomegalovirus infecties
(met een samenvatting in het Nederlands)

Proefschrift

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door

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te Ipoh, Maleisië

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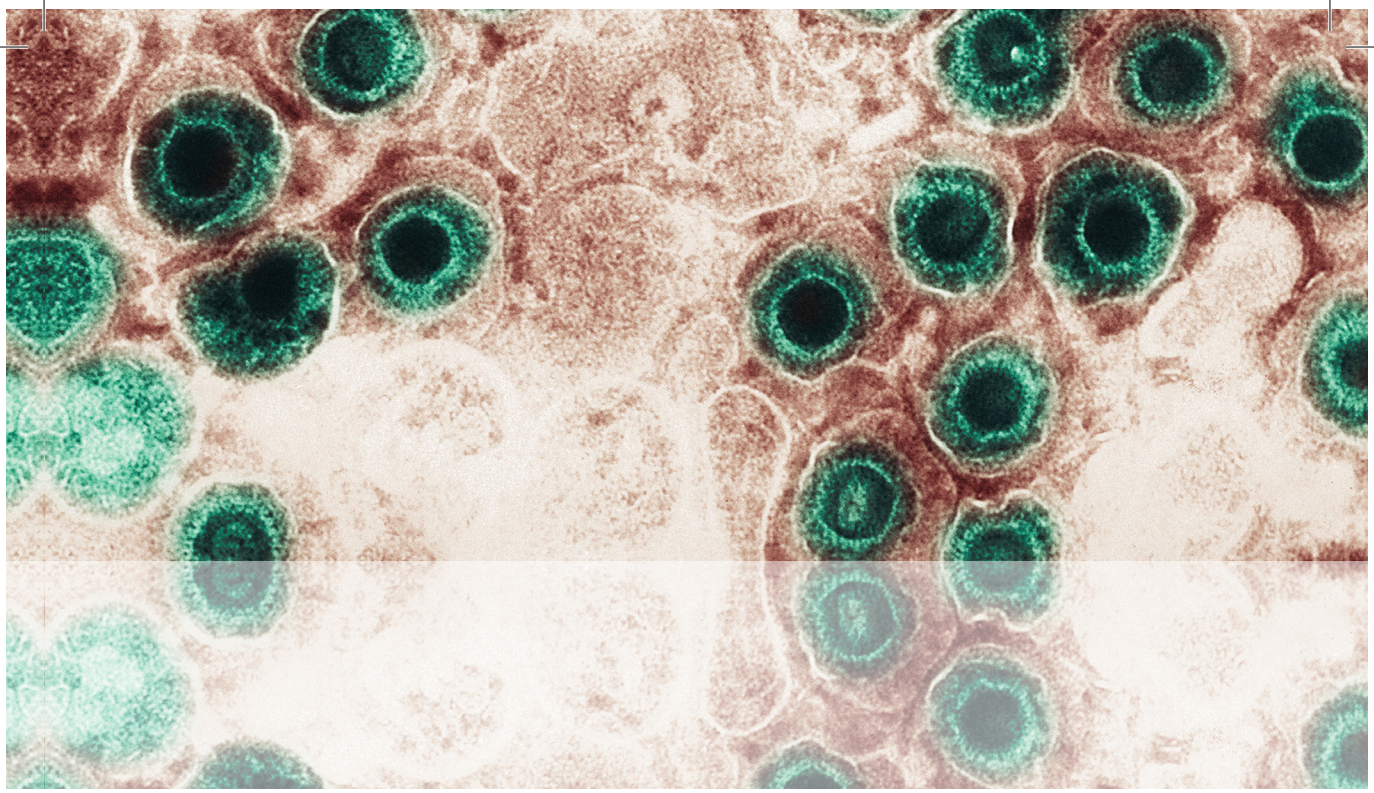
Voor Malgosia en voor alle kinderen met een congenitale of een postnatale CMV infectie

CONTENTS

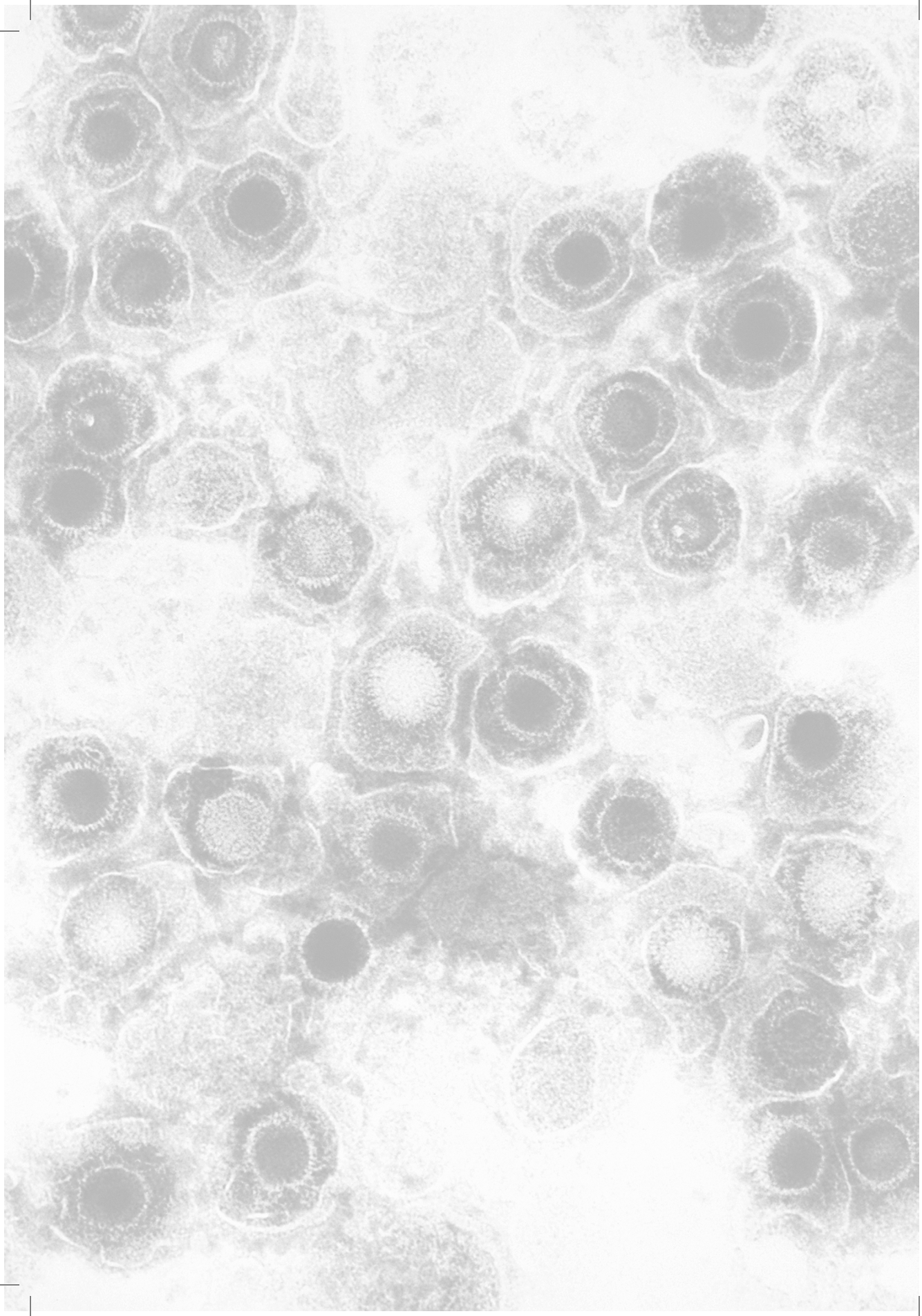


List of abbreviations

AD	Axial diffusivity
AI	Avidity index
AIOW	Age of independent walking
BSITD-III	Bayley Scales of Infant and Toddler Development-III
BW	Birthweight
CID	Cytomegalic inclusion disease
CMV	Cytomegalovirus
cCMV	Congenital cytomegalovirus
CMV-SLS	CMV-sepsis-like-syndrome
CNS	Central nervous system
CA	Corrected age
CT	Computed tomography
cUS	Cranial ultrasound
dB	Decibel
DBS	Dried blood spot card
DTI	Diffusion tensor imaging
FA	Fractional anisotropy
GCV	Ganciclovir
GA	Gestational age
GMDS	Griffiths Mental Development Scales
HC	Head circumference
Ig	Immunoglobulin
IUGR	Intrauterine growth restriction
LSV	Lenticulostriate vasculopathy
MABC-II	Movement Assessment Battery for Children-II
MD	Mean diffusivity
MRI	Magnetic resonance imaging
NEC	Necrotizing enterocolitis
NHS	Newborn Hearing Screening
NICU	Neonatal intensive care unit
PCR	Polymerase chain reaction
pCMV	Postnatal cytomegalovirus
RD	Radial diffusivity
ROI	Region of interest
RDS	Respiratory distress syndrome
SES	Socio-economic status
SGA	Small for gestational age
SNHL	Sensorineural hearing loss
TEA	Term-equivalent age
VGCV	Valganciclovir
VLBW	Very low birthweight
WM	White matter
WPPSI-III	Wechsler Preschool and Primary Scale of Intelligence-III

CHAPTER 1

General introduction and outline of the thesis



CHAPTER 1

General introduction and outline of the thesis

CHAPTER 1

1

The history of cytomegalovirus

The first account of cytomegalic cells dates back to 1881, when Professor of pathology, Dr. Hugo Ribbert first described abnormal and large, protozoan-like cells from the kidneys of a stillborn infant with congenital syphilis.[1] It was not until 1904 when Jesionek and Kiolemenoglou subsequently described similar cells found in the lungs and kidneys of an eight month old fetus with congenital syphilis, that Ribbert was able to interpret his findings.[2,3] The observations by Jesionek and Kiolemenoglou equally described protozoan-like cells with a unique nuclei, containing a "central nuclear body" encircled by a distinct halo.[2] By 1921, Goodpasture and Talbot also observed these large inclusion-bearing cells in the lungs and kidneys of a nine month old infant and attributed the cellular changes to a "metamorphosis of certain tissue cells" but they had no evidence to suggest an infectious origin. Due to the distinct and unmistakable features of the cells, they coined them "cytomegalia' (from Latin 'cyto' meaning cell, 'megalia' meaning large).[4]
After frequently detecting similar inclusion-bearing cells in lesions of herpes zoster, Lipschütz concluded that the observed cytopathic effect must be due to reactionary changes of the cell in response to an intracellular viral infection and not protozoa.[5] In 1926, Cole and Kuttner successfully inoculated young guinea pigs with an emulsion from inclusion-bearing cells of the submaxillary glands of full grown guinea pigs, noting its highly species-specific character and thus the term 'salivary gland virus' was coined.[6,7] Years before the actual causative agent was determined, Wyatt and colleagues described the fatal clinical course of six infants, in which microscopic postmortem analysis revealed the characteristic inclusion bodies in cells of the liver, pancreas, lungs, kidneys and brain.[8] They coined the term "cytomegalic inclusion disease" (CID) and were also among the first to recognize its congenital character. It was not until the virus could be propagated in vitro,[9] that Weller and colleagues were able to isolate the virus from urine and the liver of infants with CID, that they proposed the term cytomegalovirus.[10]

Cytomegalovirus

Human cytomegalovirus (CMV), also known as human herpesvirus 5, is the largest and most complex member of the herpesviridae family of viruses.[7] Human CMV is an ubiquitous beta-herpesvirus, characterized by its long replication cycle, as well as limited and species-specific host range.[11] It has a genome of approximately 230 kilobase pairs of linear double-stranded DNA.[7] CMV comprises three distinct compartments, an inner icosahedral nucleocapsid containing the viral genome surrounded by a protein-rich tegument layer and enclosed by an outer lipid-based envelope (Figure 1).[12] The tegument layer contains key antigenic stimulants such as the phosphoprotein 65 (pp65) antigen, whereas the envelope contains the classic glycoprotein complexes, the gB complex, the gM/gN complex and the gH/gL/gO complex.[12] These glycoprotein complexes elicit a neutralizing immune response, which have made recombinant versions lucrative targets for vaccine development.[13–15]

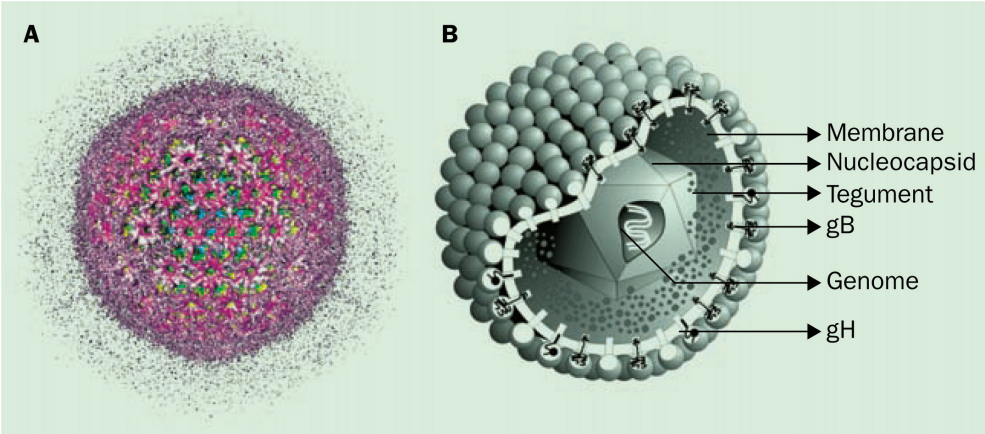


Figure 1. Cytomegalovirus. [A] Three-dimensional reconstruction of icosahedrally ordered portion of intact CMV particle as viewed along a three-fold symmetry axis. [B] Virtual three-dimensional model of CMV, depicting the various viral components. Reproduced with permission from R. Khanna.[16]

Characteristic of all primary infections of the herpesviridae family is lifelong latency within the host with intermittent reactivation and subsequent viral shedding. The complex immunological interplay involved in the mechanisms of latency and reactivation however, are still not entirely understood. Expression of unique subsets of viral transcripts ensure that the viral genome is maintained in low copy numbers in the host cell during periods of latency.[17,18] Reactivation of viral replication seems to be governed by inflammatory states in the host,[18] leading to transcriptional upregulation of major immediate-early genes in the presence of pro-inflammatory cytokines.[19] Recurrent shedding may also occur in response to reinfection with a different strain of CMV.[7] Reactivation and reinfection are also known as 'non-primary' CMV infection.[7,20] CMV is readily transmitted to mucosal surfaces and infrequently via aerosol or respiratory transmission[21] and can be transmitted through all bodily fluids, including saliva, blood, urine, amniotic fluid, cerebrospinal fluid, breast milk and secretions of the genital tract.

Infection with human CMV infection can occur in five distinct circumstances: congenital CMV infection, whereby CMV is transmitted from mother to fetus via the placenta in utero; perinatal- (infection within one month after birth) and postnatal (infection within the first 12 months after birth) CMV infection[22], whereby CMV is acquired through exposure with infected secretions in the maternal genital tract during delivery or any other exogenous contact with other infected bodily secretions, most commonly breast milk; mononucleosis or subclinical infection in otherwise immunocompetent individuals or infection in immunocompromised hosts, such as transplant recipients.

Congenital cytomegalovirus infection

Epidemiology and transmission

Congenital CMV (cCMV) infection is the most prevalent fetal viral infection worldwide with an estimated global birth prevalence of 0.7%.[23] The birth prevalence of cCMV is closely related to maternal CMV serostatus, which globally lies between 40–100% and is highest in continents like South America, Africa and Asia.[24] In the Netherlands, the CMV seroprevalence of the general population is about 45.6%, but is higher in non-Western individuals (76.7%) versus native Dutch and Western individuals (41.5%).[25] CMV can be transmitted horizontally from mother to infant at any stage of pregnancy and may be the cause of primary- or non-primary maternal infection. The overall maternal-fetal transmission rate in mothers experiencing primary infection is estimated to lie between 25–30%.[26,27] First trimester primary infections bear the lowest risk of transmission (31% versus 40% in third trimester), however pose the highest risk of symptomatic cCMV infection in the offspring.[26] Despite the highest transmission rate, third trimester infections result in mild- to no symptoms in the infant.[26,28] Pre- or periconceptional primary infections may also result in vertical transmission, however at a lower rate than in the first trimester.[26] It was previously thought that mothers who had preconceptional immunity were less likely to vertically transmit the virus and that cCMV infections that did arise under these circumstances were primarily asymptomatic at birth. Studies investigating the paradoxical correlation of high seroprevalence and high birth rates however, have postulated that non-primary maternal infections are a more frequent cause of cCMV infections probably due to reinfection with new CMV strains.[20,29,30]

Symptoms

The symptoms of a cCMV infection can range from transient and mild to severe and life-threatening. At birth, about 10–13% of affected infants will be symptomatic.[23] Approximately 80–90% of symptomatic infants will have ≥2 symptoms.[31] Symptoms can be broadly classified into three main groups: laboratory abnormalities, clinically apparent abnormalities and central nervous system (CNS) abnormalities.

Laboratory abnormalities include: thrombocytopenia, conjugated hyperbilirubinemia and elevated liver transaminases.[31,32] Clinically apparent abnormalities include: small for gestational age, petechiae, purpura, jaundice, hepatosplenomegaly[31] and pneumonia.[32] CNS abnormalities include: microcephaly, seizures, hypotonia, lethargy, chorioretinits, abnormal cerebrospinal fluid indexes or detection of CMV-DNA in the cerebrospinal fluid, sensorineural hearing loss (SNHL)[31–33] and abnormal cerebral neuro-imaging findings (intracranial calcifications, hydrocephalus, ventriculomegaly, white matter cysts, white matter signal intensity changes, migrational disorders (Figure 2).[34–37] Symptoms of cCMV infection can in some cases also already be detected by antenatal sonography or fetal magnetic resonance imaging (MRI). Detectable symptoms may be cerebral[34,38] or extra-cerebral.[38–40]

Diagnosis

Most commonly, a diagnosis of cCMV infection is made when CMV is detected in the bodily fluids within two to three weeks after birth.[41,42] A positive sample hereafter is possibly due to a perinatal or postnatally acquired CMV infection. The gold standard for diagnosing cCMV infection is culture of urine or saliva samples inoculated on human fibroblasts.[42]. This method however, is time consuming whereby detection of CMV induced cytopathic effect can only be detected around three weeks post-inoculation. With the advent of advanced diagnostic methods in the 1980s, detection of CMV-DNA by means of polymerase chain reaction (PCR) has considerably accelerated the diagnostic process.[43]. With further development of conventional PCR, real-time PCR has increased assay sensitivity[44] and allows for detection and quantification of viral DNA in various bodily fluids such as saliva,[45] plasma, dried-blood spot cards (DBS)[46,47] and amniotic fluid.[26] In the transplant population, quantification of viral DNA in patients receiving antiviral therapy allows for assessment of treatment efficacy and the potential development of antiviral resistance.[48]

Primary infection with CMV leads to activation of the humoral immune response and subsequent development of CMV immunoglobulin (Ig) M in the acute phase, followed by low avidity CMV-IgG.[49] CMV-IgM antibody determination in infants to diagnose cCMV infection has shown limited sensitivity (~70%).[42]. Testing of maternal CMV-serostatus may be performed when suspicion of fetal infection arises during gestation to assess the likelihood of vertical transmission.[50]

Prevention and treatment

To date, there is no treatment against cCMV infection that has proven to be entirely effective. Six weeks of treatment with intravenous ganciclovir (GCV) in symptomatic infants with CNS involvement has shown significantly improved- or preserved hearing at six months of age[51] and improved neurodevelopment at six- and 12 months of age.[52] Some further benefit has been observed with six months versus six weeks of treatment with valganciclovir (VGV).[33] In the study by Kimberlin et al., functional assessment of the infants' best-ear showed no improvements after six months of treatment, however overall hearing of individual ears (biological assessment) showed moderate, yet significant improvement from baseline until six and 12 months of age when correcting for CNS involvement.[33] The overall benefit of six weeks versus six months however, remains unclear especially as the endpoint viral load was comparable between both groups and blood viral load appears to be associated with the development of sequelae.[53] In the Netherlands, experts currently recommend a six week treatment regime with VGCV and/or GCV in symptomatic infants with CNS involvement for the prevention of onset- and progression of hearing loss.[54] There is currently no data to support treatment of asymptomatic infants or symptomatic infants without CNS involvement.

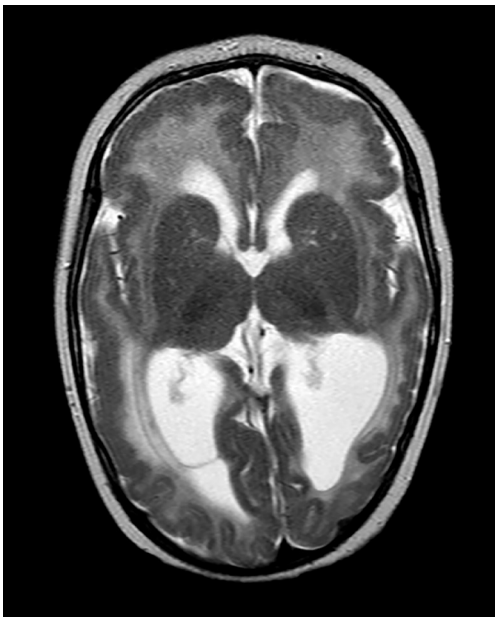


Figure 2. Cerebral MRI, axial T2-weighted sequence showing ventriculomegaly, polymicrogyria, increased signal intensity throughout the white matter, bilateral germinolytic cysts and a right occipital cyst (picture courtesy of L.S. de Vries).

Despite the lack of an entirely effective medication, knowledge of an infant's CMV status enables continued, systematic audiological follow-up for early identification and intervention of hearing loss, especially in children with late-onset hearing loss.[55–57] The age at diagnosis of late onset hearing loss is approximately 18-21 months.[56,58] In a recent longitudinal study of asymptomatic infants by Lanzieri et al., the prevalence and severity of SNHL increased throughout childhood up to 18 years of age.[57] Early intervention for hearing loss by means of hearing aids, cochlear implants and speech-language therapy can successfully impact speech- and language development, even in infants with unilateral hearing loss or severe to profound hearing loss.[58–61]

Recently, treatment of mothers with antiviral medication, carrying moderately symptomatic fetuses has shown promising results however, further investigations are necessary.[62] Ideally however, cCMV infection should be prevented with a universal vaccine. In 2000, the development of a CMV vaccine was deemed a top priority by the US Institute of Medicine.[15,63] Since then, considerable advances have been made in the field of CMV vaccine development such as identification of several potent vaccine candidates and evidence of protection.[15,64] A CMV subunit vaccine comprising gB/MF59 has shown an efficacy of 50% in a randomized, double blind, placebo controlled trial amongst young mothers; however, no conclusions

could be made regarding prevention of cCMV infection in the offspring.[65] Recently, differences in CMV neutralizing responses were demonstrated between transmitting and non-transmitting mothers in response to the CMV pentameric complex gH/gL/pUL128-131.[66] Early induction of highly neutralizing antibodies in non-transmitting mothers was associated with a reduced rate of vertical transmission.[66] Despite these promising advances, no CMV vaccine is registered yet.[15] The goal and challenge of CMV vaccine development lies in bringing together key antigens to develop a vaccine with sustained protection.[15]

Furthermore, continued efforts are need to fully understand the natural immune correlates involved in CMV transmission and to promote public awareness of cCMV infections to ensure adequate enrollment once a successful vaccine becomes available.[63,67] A lot of attention has been placed on to the potential efficacy of CMV-specific hyperimmune globulin in preventing CMV transmission in utero or preventing severe CMV disease in fetuses with cCMV infection. An initial nonrandomized study indicated a reduced rate of cCMV transmission and disease after administration of hyperimmune globulin;[68] however, these promising findings were not confirmed in a subsequent randomized controlled trial.[69]

Currently, the best form of protection against CMV is prevention of infection. Hygiene measures amongst seronegative pregnant women have shown to significantly reduce vertical transmission of CMV during pregnancy.[70] Imperative to the success of these measures is awareness and knowledge of the disease process amongst health care professionals, which is lacking considerably.[71,72]

Long-term outcome

It is estimated that children with cCMV infection suffer more from adverse effects than other well-known childhood illnesses.[73] Amongst symptomatic infants at birth, approximately 40–58% will develop one or more long-term sequelae in the form of SNHL, cognitive- and motor disabilities, cerebral palsy, epilepsy and ophthalmological abnormalities.[74–77] Approximately 9–18% of asymptomatic infants may also develop long-term sequelae usually in the form of late-onset SNHL.[23,78,79] Infant mortality rates are infrequently reported and vary between 10-30%[23] but may be less than 5%.[80]

Postnatal cytomegalovirus infection

Epidemiology and transmission

Postnatal CMV (pCMV) infections occur frequently in the neonatal population, with an estimated median incidence of around 20% (range: 6–59%).[81]. Transmission of CMV to the infant can occur through contact with any infected bodily fluids.[82–84] Most frequently however, CMV is transmitted via breast milk of seropositive mothers undergoing idiopathic local reactivation of latent CMV in the mammary gland

after birth.[85] As such, variations in the incidence of pCMV infection can be attributed to geographical and ethnical differences as well as difference in pre-treatment methods of breast milk between countries.[81] Around 96% of seropositive mothers will eventually shed CMV in their breast milk, but transmission and subsequent primary infection in the infant occurs in about 37%.[83] CMV-DNA can be detected in breast milk within the first week after birth, reaching its peak between 3–6 weeks and subsequently decreasing again to fall below detection limits by the eighth week.[86] Important independent risk factors for pCMV infection acquisition are being born to mothers of non-Western origin, which is directly related to the geographic distribution of CMV seroprevalence, as well as being fed untreated breast milk.[87]

Symptoms

A pCMV infection in most cases does not cause any symptoms and symptoms that do occur are often mild and self-limiting. Healthy term infants are unaffected.[88] Preterm infants and very low birth weight infants (VLBW) are at risk for more severe symptoms.[89] CMV-sepsis-like-syndrome constitutes the triad of apnea, bradycardia and gray pallor, and is seen as the most frequent clinical presentation occurring in about 4.5% of preterm infants and VLBW.[84] The most frequent laboratory abnormality and often only symptom of a pCMV infection is thrombocytopenia.[81] Other clinical features such as pneumonia, hepatitis and cholestasis may also occur but infrequently.[89–91] Amongst preterm infants, a pCMV infection may exacerbate pre-existing morbidity.[92] Unlike in cCMV infection, hearing does not appear to be affected.[90,93]
Neuro-imaging studies are scarce in infants with pCMV infection. The development of lenticulostriate vasculopathy (LSV) by term equivalent age as seen by cranial ultrasound, has been seen significantly more often in pCMV infected infants.[87]

Diagnosis

There is currently no gold standard in diagnosing pCMV infection, but a CMV positive sample three weeks after birth with a prior negative CMV sample is indicative of a pCMV infection.[84] A pCMV infection is nowadays most frequently tested using CMV-PCR of urine or saliva.

Treatment and prevention

To date, there are no controlled studies that have investigated treatment efficacy of antiviral medication such as GCV and/ or VGCV in infants with pCMV infection. As such, there are also no evidence-based treatment guidelines available. Efficacy studies on antiviral treatment have been carried out in symptomatic, cCMV infected, term infants[51] but these results cannot be generalized to preterm and VLBW infants with symptomatic pCMV infection. Incidental case-report studies have described the use of GCV and/or VGCV in severely, life-threatened symptomatic infants whereby administration of antiviral medication led to a reduction in CMV viral load which eventually led to normalization of clinical and hematological parameters.[85,94]

Prevention of pCMV infection in vulnerable infants can be achieved by withholding breast milk or pre-treatment by means of freeze-thawing or pasteurization. All three methods however have disadvantages. Withholding breast milk entirely, will prevent transmission but will also deprive the infant of important nutritional and immunological properties of breast milk.[95] Freeze-thawing will preserve those properties but does not entirely eliminate infectious virus,[96] whereas pasteurization will entirely eliminate the virus but also destroy important nutritional components of breast milk.[97]

Long-term outcome

There is currently no definitive consensus on the long-term outcome of pCMV infection, especially in early childhood and adolescence. Studies that have been conducted are frequently based on small sample sizes. The first study reporting on outcome by Paryani et al.[98] described pCMV infected infants that developed severe handicaps in the form of SNHL, cognitive and motor impairments as well as EEG abnormalities. Preceding studies have not observed such severe sequelae, despite occasional case reports.[99] At one and two years of age Jim et al. found no differences in hearing and development of 14 pCMV infected infants and 41 control infants.[100] Similarly, Vollmer et al. found no adverse outcome at two and 4.5 years.[101] More recent studies of late childhood and early adolescence have demonstrated minor differences in cognitive- and motor performance amongst pCMV infected children and controls but scores were still in the normal range.[93,102,103] A study using functional MRI in ex-preterm adolescents with pCMV infection, found unfavorable differences in the execution of cognitive tasks compared to ex-preterm adolescents and ex-term adolescents, suggesting a negative consequence in neurobiological processing due to pCMV infection. The authors concluded that early pCMV infection may affect higher cognitive functions, only developing later in life.[103,104]

Aims of the thesis

Congenital CMV infection has long been recognized as the leading non-genetic cause of SNHL and is increasingly associated with an adverse impact on long-term neurodevelopment in symptomatic- and also asymptomatic children. Early identification of cCMV infection is paramount to enable timely therapeutic intervention in the early stages of neurodevelopment. After recent studies have indicated an unfavorable level of general knowledge amongst health care providers in the Netherlands, several questions arose regarding the effectiveness of identifying congenitally infected infants, the uniformity of management practices and disease burden.
With the advent of improved neonatal intensive care, preterm infants are subjected to CMV at a stage of immunological immaturity and preterm associated co-morbidities. It has been hypothesized that pCMV infection in extremely preterm infants may be comparable to a fetal cCMV infection in the latter part of gestation and as such may bear similar sequelae. While full-term infants with pCMV infection seem largely unaffected,[88] there are some doubts whether cerebral development and (long-term) outcome are equally unaffected in the preterm population.

The aims of this thesis were:

1. To evaluate the recognition and management of cCMV infection.
2. To determine the disease burden of cCMV and to evaluate neuro-imaging of cCMV infected infants in relation to neuro-development.
3. To evaluate available diagnostic methods for rapid diagnoses in preterm infants with pCMV infection, neuro-imaging findings, and long-term neurodevelopmental outcome.

Outline of the thesis

Chapter 1 gives an introduction of the historical perspectives and characteristics of cytomegalovirus, congenital cytomegalovirus infection and postnatal cytomegalovirus infection.

In **Chapter 2** we assessed the general knowledge of cCMV infection amongst a select group of European experts and present data on the national cCMV registry conducted in The Netherlands.

In **Chapter 3** we presented the antenatal course of five cases of cCMV infection, their outcome and discuss the pitfalls of antenatal maternal serology interpretation.

In **Chapter 4** we demonstrated the additional value of cranial MRI compared to cUS and elucidate the relationship between timing of maternal infection during pregnancy and outcome in infants with cCMV infection.

In **Chapter 5** we reviewed the epidemiology, symptomatology and identify predictors of severity in the clinical course of infants with pCMV infection and the implications for treatment.

In **Chapter 6** we investigated the diagnostic accuracy of urine PCR versus saliva PCR in preterm infants with postnatally acquired cytomegalovirus infection.

In **Chapter 7** we used the neuro-imaging modality DTI to assess white matter microstructure and neurodevelopmental outcome at 16 months corrected age using the GMDS in preterm infants with pCMV infection.

In **Chapter 8** we evaluated neurodevelopmental outcome of preterm children with pCMV infection at 16 months corrected age using the GMDS, at 24 – 30 months of age using the BSITD-III and at 5.5 years of age using the WPPSI-III and the MABC-II.

Chapter 9 provides a summary and discussion of this thesis and directions for future research.

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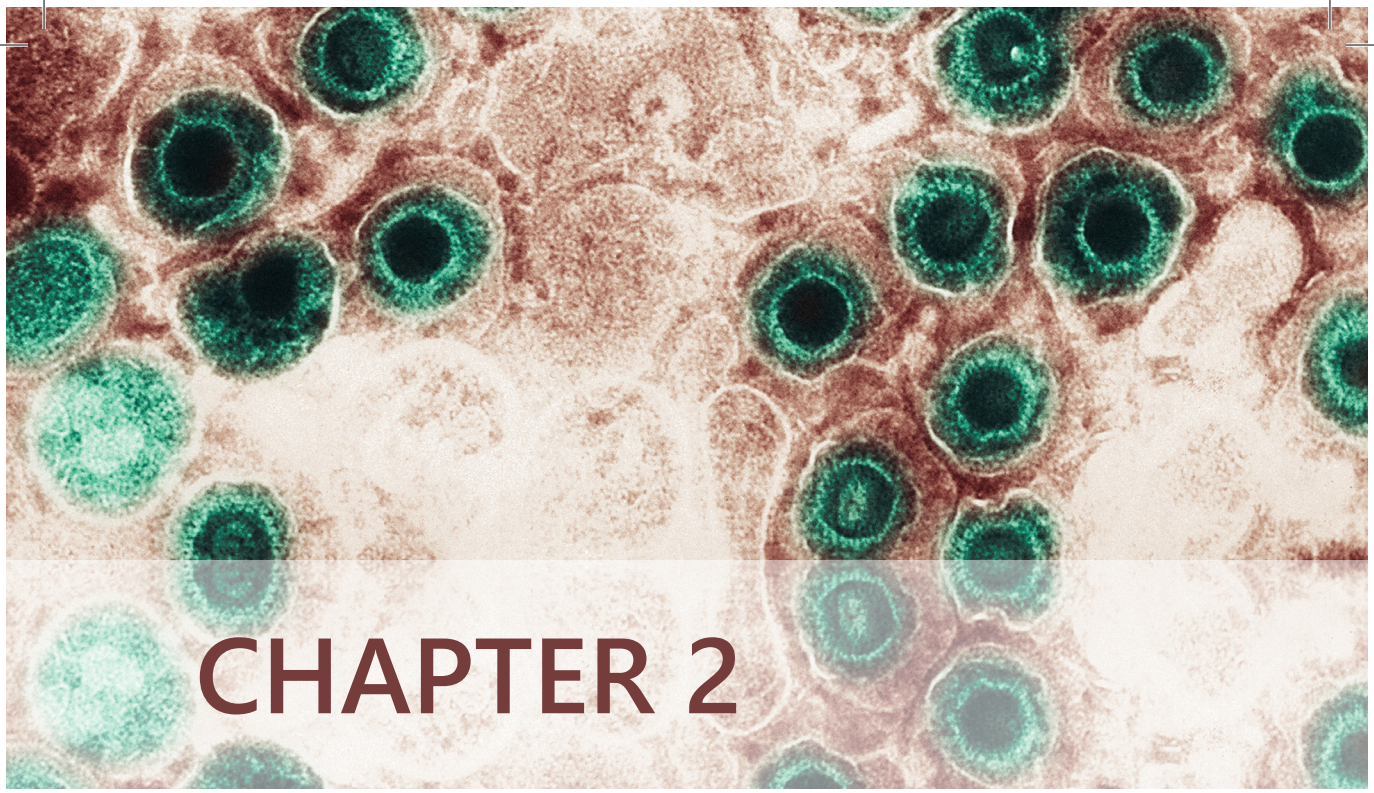

CHAPTER 2

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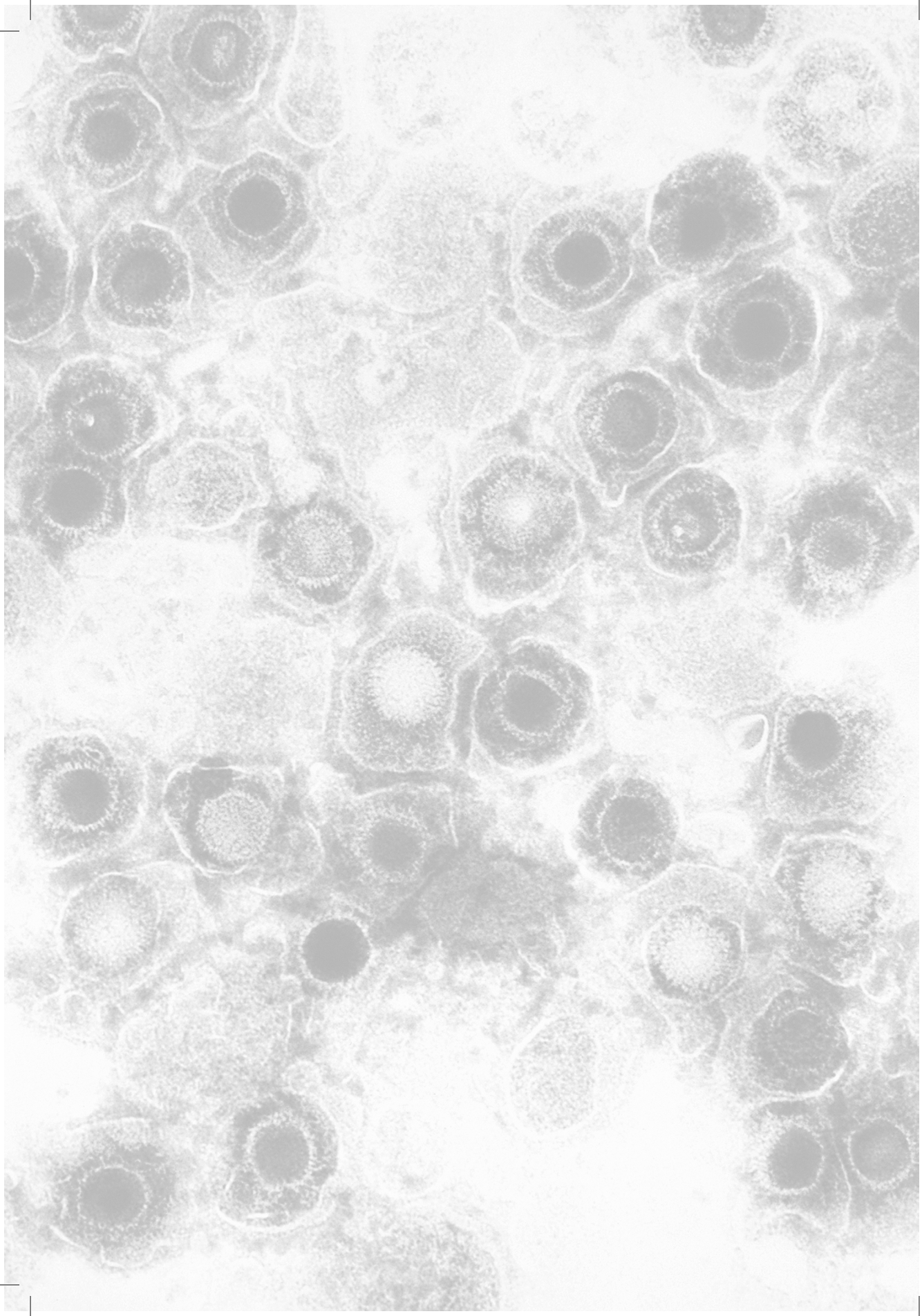

CHAPTER 2

Congenital cytomegalovirus infection in the absence of maternal CMV-IgM antibodies

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Abstract

Background Congenital CMV (cCMV) infections are the most prevalent intra-uterine infections worldwide and are the result of maternal primary- or non-primary infections. Early maternal primary infections are thought to carry the highest risk of fetal developmental abnormalities as seen by ultrasound; however non-primary infections may prove equally detrimental.

Methods/Results This case series presents five cases with fetal abnormalities detected in the second and third trimester, in which cCMV infection was considered ruled out due to negative maternal CMV-IgM.

Discussion This series highlights the possible pitfalls in serology interpretation and fetal diagnosis necessary for appropriate parental counseling. Once fetal abnormalities have been confirmed and cCMV is suspected, maternal CMV-serostatus and fetal infection should be determined. Maternal CMV-serology may be ambiguous; therefore, caution should be exercised when interpreting the results.

Introduction

Congenital cytomegalovirus (cCMV) infections occur frequently with a birth prevalence of around 1%, of which 11% of the infants will be symptomatic at birth.[1] Transmission of CMV can occur due to a primary maternal infection in previously seronegative women or after a non-primary infection (reactivation of an endogenous strain or reinfection with a new CMV strain) in women with preconceptional immunity.[2] Vertical transmission to the fetus during a maternal primary infection occurs in about 30%[3,4] and during non-primary infections in about 1-2% of pregnancies,[1] although this rate may be higher.[2] Maternal primary infections early in pregnancy occur less frequently but are thought to carry the highest risk of fetal abnormalities as seen by ultrasound (US) and/or symptomatic disease at birth.[3,4] This has not been established for non-primary infections. CMV-associated fetal US abnormalities are broadly defined as cerebral or extra-cerebral and can be transient, non-specific and may occur in any trimester. Extra-cerebral US abnormalities include intra-uterine growth restriction (IUGR), hydrops, hepatomegaly, and echogenic bowel.[5] Cerebral US anomalies can be mild (lenticulostriate vasculopathy (LSV), germinolytic cysts, mild ventricular dilatation, periventricular echogenicity) or more severe (cystic lesions in the white matter, moderate to severe ventricular dilatation, cerebellar hypoplasia, polymicrogyria and lissencephaly, microcephaly).[6-8] In the presence of cerebral abnormalities there is a high risk of adverse neurodevelopmental outcome.[7,9] Primary infections are recognized by CMV-IgG seroconversion or positive CMV-IgM with a low IgG avidity index (AI).[1]

When looking at CMV-IgM kinetics following primary infection, peak levels are seen in the first 1-3 months, after which the titers begin to decrease.[10] Occasionally, persistent (low) levels of CMV-IgM can be detected >3 months or up to a year.[10] Non-primary infections are difficult to diagnose but may be recognized by positive CMV-IgG prior to conception/ early gestation in combination with a positive CMV-IgM and CMV-IgG with high IgG-AI and/or a significant increase in CMV-IgG titer during gestation.[1,10,11] When the type of maternal infection cannot be classified on the basis of CMV-IgM, CMV IgG-AI may aid in distinguishing between primary and non-primary infection.[13,19] A high IgG-AI is usually seen around 5-6 months following primary infection. Dating the timing of the infection through maternal serology is difficult and interpretation of the results is not always straightforward, especially because samples are often collected long after the maternal CMV infection has occurred. Here we report five cases with fetal anomalies in the second and third trimester, whereby cCMV infection was initially considered unlikely because maternal CMV-IgM was negative at the time of presentation.

Case results

Case 1 was referred at 20 weeks gestation (WG) because of fetal echogenic bowels. Amniocentesis and maternal serology testing for CMV and toxoplasmosis were offered but declined. Genetic carrier testing of both parents revealed no mutation on the CFTR gene and therefore cystic fibrosis was considered unlikely. The US at 32 WG to monitor bowel development showed reduced echogenicity but now revealed extensive bilateral LSV (Figure 1A). Maternal CMV-serology was tested at this time and was indicative of CMV infection in the past (Table 1). At 40+1 WG a female infant was born with a birth weight (BW) of 3460 grams (percentile (p) 45), length 52 cm (p50), a head circumference (HC) of 36 cm (p50) and Apgar scores of 8/10/10. The infant had widespread petechiae, hepatosplenomegaly and thrombocytopenia ($44x10^9$/l). Cranial US on day of life (DOL) 1 indicated extensive bilateral LSV, white matter calcifications and bilateral germinolytic cysts. Urine CMV polymerase chain reaction (PCR) tested positive confirming cCMV infection. Hearing and ophthalmological tests were normal. The infant was treated with valganciclovir for six weeks. MRI performed at five months of age showed white matter signal intensity changes and resolution of the germinolytic cysts. First trimester maternal serum was tested in retrospect and was indicative of a primary CMV infection (Table 1). The Alberta Infant Motor Scale and Bayley Scales of Infant and Toddler Development (BSITD-III) at 12 months of age showed mild neurodevelopmental impairment.

Case 2 was referred at 30+2 WG due to mild bilateral ventriculomegaly. This was a repeat US after a routine US at 20 WG showed mild hydronephrosis, which had now normalized. Maternal serology was negative for toxoplasmosis and indicated CMV infection in the past (Table 1). The dilatation was initially progressive and eventually stabilized at 35 WG. At 38 WG a female infant was born with a BW of 2750 grams (p20) length of 54 cm (>p97), a HC of 33 cm (p20), Apgar scores of 8/ 9/10 and no physical symptoms. Laboratory analysis revealed thrombocytopenia ($114\ x10^9$/l), resolving spontaneously by DOL 8. Cranial US performed on DOL 1 showed bilateral ventriculomegaly, bilateral LSV, germinolytic cysts and a cyst in the right temporal lobe. MRI was carried out on DOL 2 and showed high signal intensity in the white matter, bilateral: germinolytic cysts, subependymal cysts, large temporal cysts and small occipital cysts. Urine CMV-PCR tested positive on DOL 2 confirming cCMV infection. Hearing and ophthalmological tests were normal. The infant was treated with valganciclovir for six weeks. First trimester maternal serum could not be tested retrospectively as it was discarded. The Griffiths Mental Developmental Scales (GMDS) assessment at 36 months was within normal range (developmental quotient 100) and her hearing was normal.

Case 3 was referred at 21+4 WG due to a HC<p3, femur length (FL)<p3, an enlarged heart, oligohydramnion and echogenic bowels. Advanced US confirmed these findings and additionally revealed fetal hydrops and a thickened nuchal fold. Maternal serology was tested and was negative for toxoplasmosis, enteroviruses, parvovirus B19, varicella zoster virus and showed CMV infection in the past (Table 1). Amnio-

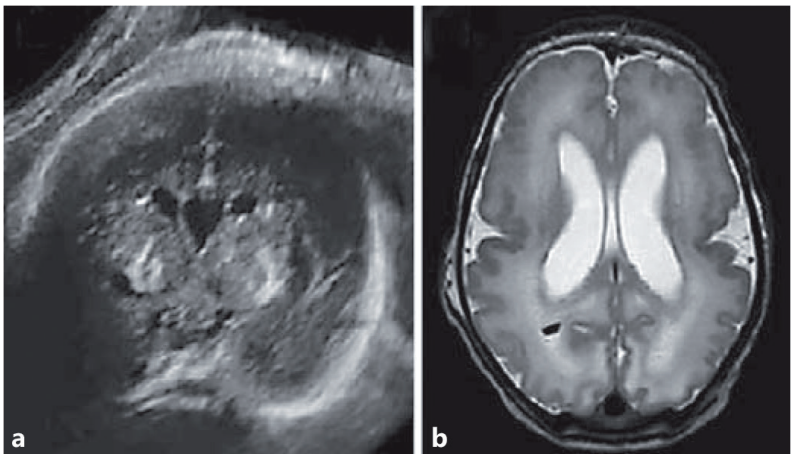

Figure 1. [A] Case 1: fetal US at 32 weeks gestation, coronal plane, showing bilateral LSV; [B] Case 5: MRI, axial T2 weighted sequence, showing ventriculomegaly, increased signal intensity throughout the white, a small hemorrhage in the white matter (right occipital) and bilateral extensive polymicrogyria.

centesis was performed at 22+4 WG revealing a genetic duplication and deletion, which was also present in the mother and therefore not considered causative. CMV-PCR was not carried out at this point. Fetal growth stagnated at 23+3 WG with HC <p3 and increase in fetal hydrops. Parents were counseled about the risk of a poor outcome and choose to terminate the pregnancy. At 23+6 WG a male infant was born weighing 573 grams (p10-20). Autopsy confirmed the pericardial effusion and ascites but not IUGR (HC p20-50, FL p20-50). The heart had a dilated left ventricle and a small atrium-septum defect. Immunohistochemical staining was positive for CMV in the pancreas, spine, liver, lung, kidneys and placenta and severe cytomegalic inclusion bodies were found throughout the brain, confirming cCMV infection. Retrospective analysis after fetal autopsy, of first trimester maternal serum and amniotic fluid revealed CMV infection in the past and a positive CMV-PCR, respectively. Unfortunately a CMV-IgG AI was not possible to determine as no serum was available for additional tests.

Case 4 was referred at 21+6 WG for a HC <p5. Advanced US revealed a HC at p3 and cerebellar hypoplasia (transcerebellar diameter (TCD) <p3). Second trimester maternal serology was negative for toxoplasmosis and indicated CMV infection in the past (Table 1). Amniocentesis ruled out chromosomal abnormalities, CMV-PCR was not performed. A repeat US at 22+5 WG showed persistence of the HC <p3 and cerebellar hypoplasia (<p3). Parents were counseled about the poor prognosis and decided to terminate the pregnancy. Autopsy revealed a female infant weighing 595 grams (p20-50), with no exterior abnormalities. The small cerebellum was confirmed alongside a small brain (p5). Microscopic examination showed CMV inclusion bo-

dies in the kidneys, pituitary gland and throughout the cerebellum and cerebrum, confirming cCMV infection. Due to the discrepant results between second trimester maternal serology and autopsy findings, first trimester- (in retrospect) and postpartum maternal serum were tested for CMV. First trimester maternal serum revealed a possible non-primary infection with positive CMV-IgM and a high IgG-AI (Table 1). Postpartum serology was the same as in the first trimester sample however with an increase of the IgG-AI.

Case 5 was referred at 22+2 WG for a HC <p3, echogenic bowels and oligohydramnion. Advanced US confirmed these findings. Maternal serology was tested at 22+2 WG revealing CMV infection in the past (Table 1) and no signs of toxoplasmosis, rubella virus, Treponema pallidum or varicella zoster virus. Amniocentesis was declined and the parents decided to continue the pregnancy. Throughout the pregnancy, HC and cerebellar growth remained below the p3. At 37+4 WG a female infant was born weighing 2890 grams (p20-50), length 47 cm (p10-20), HC 32 cm (p3) and Apgar scores of 7/7/9. The infant had widespread petechiae, purpura, hepatosplenomegaly, thrombocytopenia ($30x10^9$/l), conjugated hyperbilirubinemia (total 455 umol/l, direct >170 umol/l) and prolonged partial thromboplastin (PT) and activated thromboplastin time (APTT) (PT: 25.0 sec; APTT 51 sec). Cranial US on DOL 1 showed mild bilateral ventriculomegaly, a smooth aspect of the cortex and bilateral LSV. MRI was performed on DOL 3 showing extensive polymicrogyria, intraventricular hemorrhage, subdural hemorrhage, supra- and infratentorial hemorrhagic lesions in the white matter and multiple punctate hemorrhages in the cerebellum (Figure 1B). Urine CMV-PCR on DOL 1 was positive, confirming cCMV infection. Ophthalmological examination revealed lesions suggestive of chorioretinits in one eye. Hearing was not tested. Clinical condition deteriorated on DOL 3. Due to the poor prognosis, intensive care was not intensified and the infant died. Postmortem examination was declined. First trimester serum was retrospectively tested and indicated CMV infection in the past (Table 1).

Discussion

This series describes fetal anomalies suggestive of CMV infection whereby maternal serology at the time of anomaly detection was CMV-IgM negative. Vertical CMV transmission was therefore considered unlikely in all cases. In the Netherlands, pregnant women are not screened for CMV but are routinely tested in the first trimester for HIV, syphilis and hepatitis B. At 20 WG a routine sonogram is offered. When abnormalities are found, women are referred for an advanced US and, when needed, further diagnostics such as maternal serology testing and/or amniocentesis are offered. A pre- and postnatal national guideline is available when CMV infection is suspected. In practice, however, when maternal serology is suggestive of a CMV infection in the past due to absence of CMV-IgM, the possibility of vertical transmission is often ruled out. This series highlights the inhomogeneous presentation of maternal CMV-serology and stresses the need for cautious interpretation and warrants

multiple diagnostic steps.[12] In all cases the diagnosis of cCMV infection was made postpartum due to infant symptomatology or fetal autopsy findings. A crucial, albeit obvious learning point lays in the fact that in all cases maternal CMV-serology was only tested once when anomalies were first detected. Intra-uterine infections are an important differential group when fetal anomalies arise and are frequently tested by examining maternal serology for the TORCH-complex (toxoplasma, other infectious pathogens such as rubella virus, cytomegalovirus and herpes viruses). When considering CMV-serology, however, factors such as gestational age at testing are essential to correct interpretation. Despite extensive literature on the topics of maternal CMV-serology (for a review see 12, 13) and CMV-induced fetal anomalies,[4,5,13] in our experience the awareness of CMV as potential cause of fetal anomalies has not sufficiently penetrated clinical practice.[14,15]

For correct interpretation of maternal CMV-serology it is ideal to know the CMV-serostatus antecedent to pregnancy. Since this is often unknown, serum samples from standard screening (HIV, syphilis and hepatitis B) in the first trimester should be saved to enable retrospective analysis of potential early primary infection. Frequently, serum samples are discarded immediately making retrospective analysis not possible. Unfortunately, this was the case in case 2 and therefore it was not possible to discern the type of maternal infection. The serology in case 1 exhibits the classic characteristics of a primary infection with low IgG-AI and a positive CMV-IgM in the first trimester. When symptoms are evident in the child after birth, we advise to immediately (no later than ≤3 weeks postpartum) perform CMV-PCR on the infants' urine to determine if vertical transmission has occurred. Case 3 may also exhibit a primary infection despite negative CMV-IgM in the first trimester. At 8+3 WG, two months have passed in which CMV-IgM could have already dropped below the detection limit.[11] Unfortunately not enough serum was available for a first trimester IgG-AI test. However, an IgG-AI of 0.74 (VIDAS, bioMérieux high IgG-AI >0.65) at 21+4 WG could support an early primary infection. IgG-AI is low during the first 3-4 months after primary infection, followed by an intermediate IgG-AI for 1-2 months and subsequently full IgG avidity maturation, fitting this timeline.[11] In both cases the decision to terminate the pregnancy may have not been altered; however, we believe that when clinicians choose to test for CMV that this should be done correctly to accurately counsel parents.

It was previously thought that non-primary infections have a low transmission rate due to preconceptional immunity, however, more recently it has been noted that cCMV infections could occur more often in infants of preconceptionally seropositive women (for a review see 2, 16). Severe CMV-associated symptoms in the fetus/infant, as a result of non-primary maternal infection have been reported[17] and is the probable cause in cases 4 and 5. Serological characteristics of non-primary infections remain elusive;[18] however, when fetal anomalies are present and maternal serology indicates non-primary infection it is important to realize that cCMV infection may still have taken place. A high IgG-AI regardless of the presence of CMV-IgM reflects immunological maturity and encountered early on in gestation pleads against first trimester primary infection.[12,18] Unfortunately, not enough serum was available for a

Table 1. Characteristics of five fetuses with ultrasound findings suggestive of CMV infection and maternal serology.

Case	Maternal	GA at first presentation	Fetal US anomalies	GA at testing	CMV serology at time of US (2T/3T)			GA at testing	CMV serology at 1T			Interpretation
					IgM	IgG	Avidity		IgM	IgG	Avidity	
1	G4P2	20	Echogenic bowels, LSV (at GA 32)	32	-	+	0.88[a]	12	+	+	0.27[a]	Primary infection
2	G2P1	30+2	VM	32+4	-	+	0.95[a]	NA	NA	NA	NA	Unknown
3	G2P1	21+4	HC<p3, FL<p3, enlarged heart, oligohydramnios, echogenic bowels, FH, thickened nuchal fold	21+4	-	+	0.74[a]	8+3	-	+	NA	Probable primary infection
4	G2P1	21+6	HC p3 Cerebellum<p3	21+6	-	+	0.68[a]	13	+	+	0.62[b]	Non-primary infection
5	G1P0	22+2	HC <p3 Echogenic bowel	22+2	-	+	0.96[a]	12	-	+	0.91[a]	Non-primary infection

CMV: cytomegalovirus; GA: gestational age in weeks+days; 1T: first trimester; 2T: second trimester; 3T: third trimester; IgM: Immunoglobulin M; IgG: Immunoglobulin G; HC: head circumference; FL: femur length; LSV: lenticulostriate vasculopathy; US: ultrasound; VM: ventriculomegaly; FH: fetal hydrops; NA: not available.
[a] VIDAS, bioMérieux CMV IgG avidity index (AI; high AI: >0.65, intermediate AI: 0.65-0.40, low AI: <0.40); [b] LIAISON XL, DiaSorin CMV IgG AI (high AI: >0.25, intermediate AI: 0.25-0.15, low AI: <0.15).

retrospective CMV-IgM analysis in case 4, however the very high IgG-AI at 11+5 WG points to a non-primary infection regardless of CMV-IgM. When interpreting IgG-AI amongst different laboratories, it is important to be aware of different AI-cut-off values between assays (case 4).[19] To ensure diagnostic accuracy, it is advisable to test all samples in one laboratory. IgG-AI testing in case 4 was done in different laboratories due to referrals.

A useful gestational management scheme for CMV infection is proposed by Lazzarotto et al.[12], but since maternal CMV screening is not felt justified in most countries, a frequent diagnostic starting point are fetal anomalies seen on routine 20 WG sonograms. Fetal infection can be diagnosed by CMV-PCR of amniotic fluid and has been shown to have good sensitivity and a low risk of fetal loss (<1%) when carried out after 20-21 WG and ≥6-8 weeks after the onset of maternal infection (if known).[4,10,12] When amniocentesis is performed for other diagnostics (i.e. quantitative fluorescence-PCR), we recommend to concurrently perform CMV-PCR to investigate fetal CMV infection. This would have accelerated the diagnosis in cases 3 and 4. Despite the fact that the clinical course may have not changed significantly, we want to stress the importance of CMV as a causative agent of fetal abnormalities and that when testing is done, that this should be carried out at the correct time points and interpreted with sufficient expertise.

In conclusion, multiple diagnostic steps should be carried out to diagnose fetal cCMV infection. When fetal US anomalies are detected, referral for an advanced US should take place. Both maternal first and second/third trimester serum should be tested for evidence of a primary infection. Storage of first trimester serum should be obligatory to enable this. In the case of negative CMV-IgM, a non-primary infection cannot always be excluded. If the mother is seronegative, cCMV infection can be excluded. To confirm fetal infection, amniocentesis can be offered. When cCMV infection is confirmed and the extent of cerebral and extra-cerebral abnormalities is determined, parents can be counseled accordingly.

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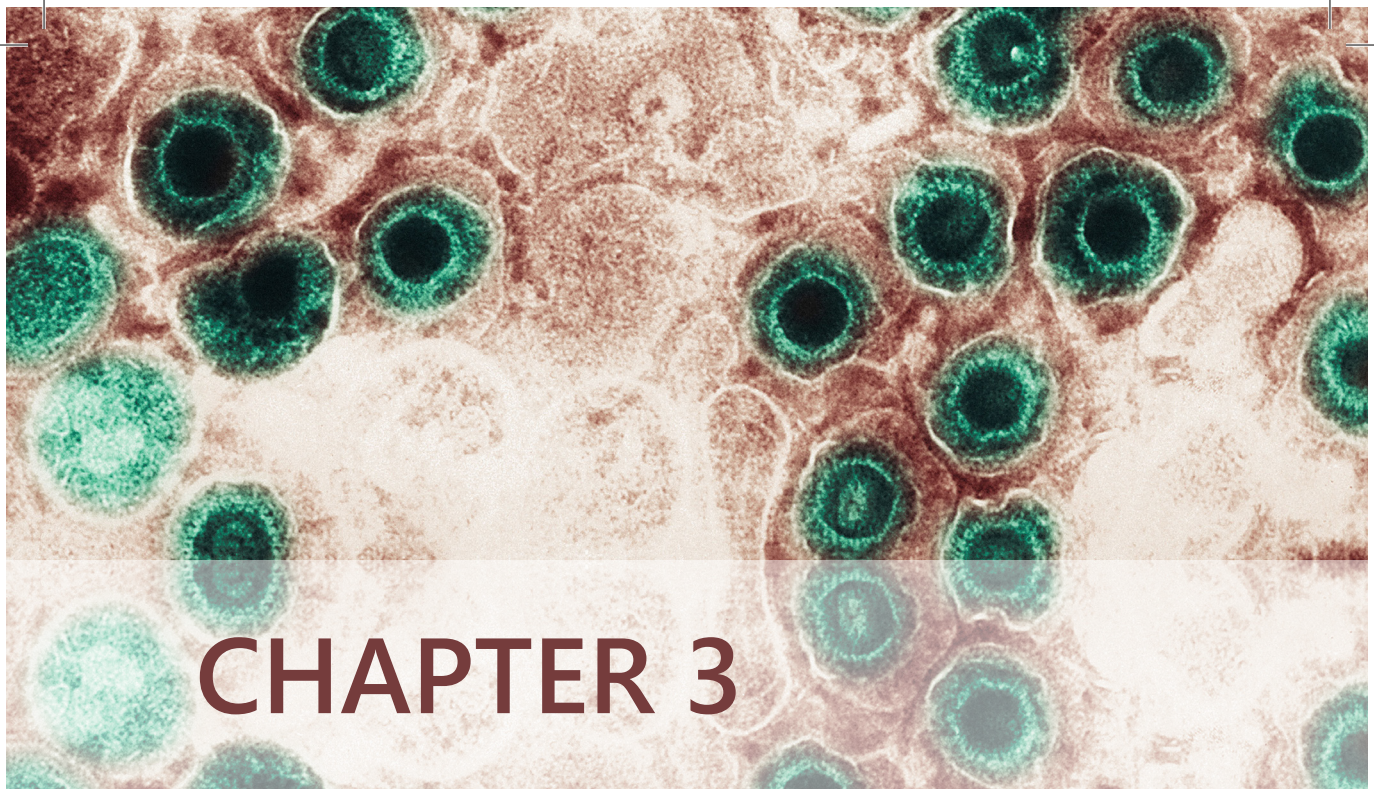

CHAPTER 3

Neuro-imaging findings in infants with congenital cytomegalovirus infection: relation to trimester of infection

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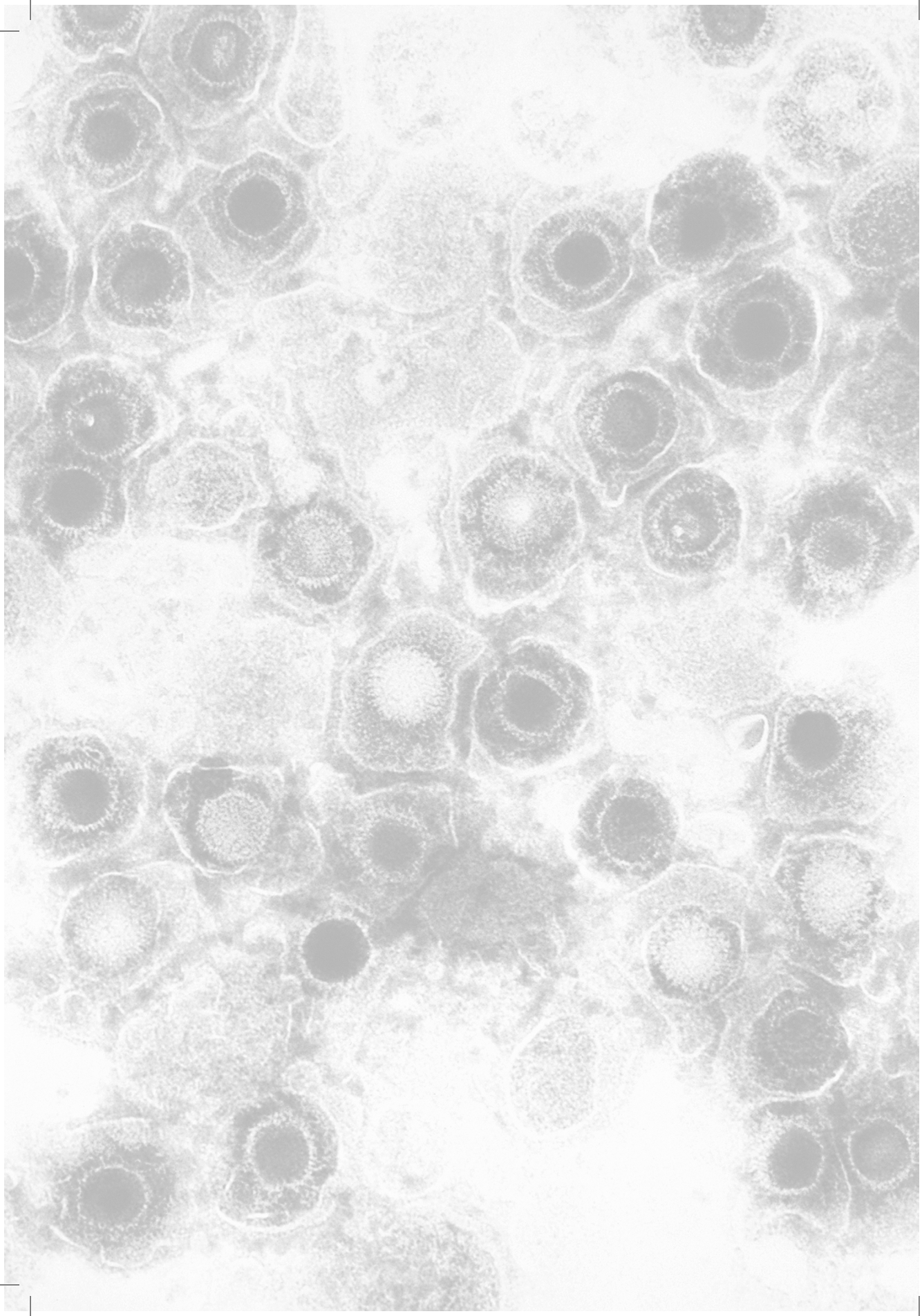

CHAPTER 3

Neuro-imaging findings in infants with congenital cytomegalovirus infection: relation to trimester of infection

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Abstract

Background Congenital cytomegalovirus (cCMV) infection early in pregnancy may result in major disabilities. Cerebral abnormalities detected using cranial ultrasound (cUS) and magnetic resonance imaging (MRI) have been related to neurological sequelae.

Objective To evaluate the additional value of MRI and assess the relationship between time of infection during pregnancy and outcome in infants with cCMV infection.

Methods and study design Demographic and clinical data were collected in infants with cCMV infection (1992–2013). Trimester of infection, neuro-imaging results and outcome were reviewed. Cerebral abnormalities were categorized into none, mild (lenticulostriate vasculopathy, germinolytic cysts, high signal intensity on T2-weighted images) and severe (migrational disorder, ventriculomegaly, cerebellar hypoplasia). Results were statistically analyzed.

Results Thirty-six infants were eligible for analysis. cUS was performed in all and cranial MRI in 20 infants. Migrational disorders were only diagnosed using MRI ($p<0.01$). In 17 infants trimester of infection was ascertained. Seven out of 10 infants infected during the first trimester had severe abnormalities on cUS (five confirmed on MRI) and adverse sequelae; three had no/mild abnormalities on cUS/MRI and normal outcome. Two out of thee infants infected during the second trimester with no/mild abnormalities on cUS/MRI had normal outcome; one with mild cUS and MRI abnormalities developed sensorineural hearing loss. Four infants infected during the third trimester with no/mild abnormalities on cUS/MRI had normal outcome.

Conclusion Infants with a first trimester cCMV infection are most at risk of severe cerebral abnormalities and neurological sequelae. MRI, and not cUS, enables an early diagnosis of migrational disorders, which can improve prediction of outcome.

Introduction

Cytomegalovirus (CMV) infection is the most common fetal viral infection worldwide with an incidence of 0.2–2%.[1] About 10% of infants with a congenital CMV (cCMV) infection have clinical signs of infection at birth, such as being small for gestational age or having microcephaly, convulsions, hepatitis, conjugated hyperbilirubinemia and hematological disorders like thrombocytopenia.[1] Neonatal mortality rates due to hepatic dysfunction, hemorrhage or disseminated intravascular coagulation of symptomatic infants range between 20 and 30%.[2] Furthermore, 50–90% of the survivors develop neurological sequelae including cerebral palsy and sensorineural hearing loss (SNHL).[3] Most asymptomatic infants have a normal neurodevelopmental outcome, although SNHL may occur at a later age in 10–15%.[4] Intrauterine CMV infection can occur during any trimester of pregnancy. It has been documented that an infection early in pregnancy is more likely to be associated with a poor outcome.[3,4] In contrast, cCMV infection during the third trimester of pregnancy is unlikely to be associated with adverse sequelae.[4,5] A variety of cerebral abnormalities has been described in infants with cCMV infection using cranial ultrasonography (cUS) and magnetic resonance imaging (MRI). A relationship between severe cerebral abnormalities and neurological sequelae has been shown.[6-12]
In this study, we investigated the relationship between the time of onset of infection during pregnancy, neonatal neuro-imaging data and neurodevelopmental outcome in infants with cCMV infection.

Patients and methods

Study population

All infants diagnosed with cCMV infection between 1992 and 2013 and admitted to the level 3 neonatal intensive care unit (NICU) or maternity unit of the University Medical Center Utrecht, the Netherlands, were included. cCMV infection was confirmed by positive CMV-PCR of urine collected within 3 weeks after birth.[13] When an infant died before urine was collected, the Guthrie card was used to confirm a cCMV infection. Since 2007, all preterm (<32 weeks of gestational age) infants admitted to our NICU were screened for CMV infection as part of routine clinical care.[14]

Ethics

No permission was required from the hospital's medical ethics committee for this retrospective, anonymous data analysis.

Study design

Maternal and infant data were collected. Maternal data included: anti-CMV IgG and IgM titer, anti-CMV IgG avidity test results, results of CMV-PCR in amniotic fluid, primary infection and trimester of pregnancy during which infection occurred. The trimester of CMV infection was determined with serological and CMV-PCR test results.

When test results were inconclusive, the trimester of infection was reconstructed when possible based on the history of an active CMV infection during pregnancy. Infant data included: clinical symptoms of cCMV infection (small for gestational age and/or microcephaly, as defined by the Dutch Perinatal Registry[15]), hepatosplenomegaly, jaundice, hypotonia, convulsions, petechiae), laboratory abnormalities (thrombocytopenia, with thrombocytes $<150\times10^9$/l; conjugated hyperbilirubinaemia, with conjugated bilirubin >10 µmol/l; hepatitis, with aspartate aminotransferase >45 U/l, alanine aminotransferase >35 U/l), chorioretinitis (ophthalmological examination), SNHL at birth (automated auditory brainstem response) and CMV load in urine. Infants with >1 clinical symptom of cCMV infection and/or laboratory abnormality and/or SNHL at birth were classified as symptomatic.

Neuro-imaging

Cranial ultrasonography

All infants underwent cUS following admission to the NICU (n = 25) or maternity ward (n = 3) at the University Medical Center, Utrecht, or to the neonatal unit at local hospitals before referral to our hospital (n = 8). Performing cUS is part of routine clinical care in our NICU and was performed in preterm infants and newborns with clinical signs of cCMV infection in the maternity ward. cUS was performed using an ATL-UM4 mechanical sector scanner (Philips Healthcare, Best, the Netherlands) or an Aplio XG scanner (Toshiba Medical System, Zoetermeer, the Netherlands) with a multifrequency transducer (5–8 MHz) to ensure the best possible resolution. Abnormalities seen on cUS were categorized into 3 groups: no abnormalities, mild abnormalities (lenticulostriate vasculopathy (LSV), germinolytic and subependymal pseudocysts) and severe abnormalities (periventricular white matter calcifications, ventriculomegaly, cerebellar hypoplasia and frontal/temporal/occipital cysts in the white matter). In clinically stable infants with a symptomatic cCMV infection at birth and abnormalities on cUS, MRI was subsequently performed. Urine CMV-PCR was performed in all infants with LSV and/or germinolytic cysts or pseudocysts on cUS without clinical signs of cCMV infection.

Magnetic Resonance Imaging

MRI was performed on a 1.5- or a 3-tesla whole body scanner (Achieva; Philips Healthcare) using a head coil. The MRI contained sagittal and axial or coronal T1- and T2-weighted images. The MRI was mostly performed during the neonatal period (n=19), and when patients were referred from local hospitals MRI was performed as soon as possible (n=1). MRI results were categorized into 3 groups: no abnormalities, mild abnormalities (high signal intensity white matter on T2-weighted imaging, germinolytic cysts) and severe abnormalities (polymicrogyria, lissencephaly, hippocampal dysplasia, ventriculomegaly, cerebellar hypoplasia and frontal/temporal/occipital white matter cysts). cUS and MR images were reviewed by neonatologists with >10 years of experience in neuro-imaging.

Statistics

The association between cUS and MRI findings was assessed by means of Fisher's exact test. A p value <0.05 was considered statistically significant.

Follow-up

The developmental outcome was determined using the Griffiths Mental Developmental Scales (GMDS)[16] at 16–18 months of age and Bayley Scales of Infant and Toddler Development-III (BSITD-III)[17] at 24 months of age. The GMDS and BSITD-III give a total developmental quotient (mean 100, standard deviation 15). An abnormal developmental quotient was defined as –1 standard deviation (developmental quotient ≤ 85) in both tests. If neurodevelopmental outcome was very poor, the GMDS and BSITD- III could not be performed, and outcome was defined as adverse. Infants <12 months of age were excluded from the analysis of neurodevelopmental outcome. Hearing was assessed using auditory brainstem-evoked response examinations at 6 and 12 months post-partum and subsequently annually until 5 years of age. In infants with SNHL, the hearing loss was described separately, and the use of hearing aids or cochlear implants was recorded. Both the GMDS and BSITD-III have a separate subscale for speech and language development.

Results

Forty-four infants were diagnosed with cCMV infection. Eight (18%) infants were excluded from analysis of whom 6 due to lack of data on trimester of infection in combination with no data on neuro-imaging results, one due to severe congenital abnormalities at birth in the absence of a clear relationship with cCMV infection and one because of severe intracranial hemorrhage on cUS which was most likely due to a proven COL4A1 mutation. Baseline characteristics are summarized in Table 1. Twenty-six of the 36 included infants (72%) presented with symptomatic cCMV infection at birth. Of 10 asymptomatic infants, 3 (30%) were screened for cCMV infection as LSV or germinolytic cysts were found on cUS, in 4 (40%) CMV-PCR in urine was performed because of a maternal CMV seroconversion during pregnancy, 2 (20%) were part of a twin and quadruplet, respectively, and were screened because of a symptomatic sibling, and 1 (10%) was diagnosed with cCMV using the Guthrie card after CMV infection was diagnosed at term-equivalent age.

Neuro-imaging

Asymptomatic infants

No abnormalities were seen on cUS in 3/10 (30%) infants and mild abnormalities in 7/10 (70%).

Symptomatic infants

No abnormalities were seen on cUS in 2/26 (8%) infants, 9/26 (34%) had mild abnormalities and 15/26 (58%) had severe abnormalities. MRI was performed in 20/26 symptomatic infants; 7 (35%) infants showed mild abnormalities, and 13 (65%) showed severe abnormalities. MRI could not be performed in 3 infants as they were too unstable to be transported to the MR unit; 2 of these infants died within 1 day after birth, 1 infant died after 3 months because of severe liver failure due to CMV-related hepatitis. In 3 infants, no MRI was performed since no abnormalities were seen on cUS. Whereas LSV could only be detected using cUS, neuronal migrational disorders (9/20, $p < 0.001$) could be detected exclusively by MRI (Table 2).

Table 1. Demographic characteristics of 36 infants with congenital CMV infection and maternal characteristics.

Maternal characteristics	
Primigravida[a]	10 (28)
Non-Caucasian nationality mother	7 (19)
Caesarean section	17 (47)
First trimester infection	10 (28)
Second trimester infection	3 (8)
Third trimester infection	4 (11)
Unknown trimester infection	19 (53)
Infant characteristics	
Gestational age (weeks), mean (range)	36.6 (28– 42)
Prematurity	15 (42)
Male gender	14 (39)
Apgar score at 1 minute, median (range)	8 (1–9)
Apgar score at 5 minutes, median (range)	9 (1–10)
Symptomatic CMV infection	26 (72)
Antiviral treatment	5 (14)
Died	6 (17)

Data are presented as numbers with percentages or mean/medians with ranges in parentheses.
[a] In 4 mothers, data about obstetric history were unknown.

Table 2. Results of cUS and MRI in 20 patients with congenital CMV infection.

	cUS	MRI	P value
No abnormalities	0 (0)	0 (0)	1.000
Mild abnormalities	8 (40)	7 (35)	1.000
Germinolytic cysts	13 (65)	8 (40)	0.205
Lenticulostriate vasculopathy	16 (80)	0 (0)	**<0.001**
White matter signal intensity abnormalities	1 (5)	17 (85)	**<0.001**
Severe abnormalities	12 (60)	13 (65)	1.000
Polymicrogyria	0 (0)	9 (45)	**0.001**
Periventricular calcifications	6 (30)	2 (10)	0.235
White matter cysts	5 (25)	7 (35)	0.731
Cerebellar hypoplasia	3 (15)	6 (30)	0.451
Ventriculomegaly	11 (55)	11 (55)	1.000

Data are presented as numbers with percentages in parentheses.

Neuro-imaging results in relation to neurodevelopmental outcome

Asymptomatic infants
None of all 10 asymptomatic infants with no or mild (germinolytic cysts, LSV) abnormalities on cUS developed sequelae between 1.5 and 5.4 years of age (Table 3).

Symptomatic infants
One out of 26 infants without cUS abnormalities had a normal outcome at 2 years of age. Of 9/26 infants with mild abnormalities on cUS and MRI (germinolytic cysts, LSV, high T2-weighted signal intensity on MRI), 7/9 had a normal outcome between 1.1 and 3 years of age and 2/9 had bilateral SNHL at birth; 1 infant had hearing thresholds of 45 and 90 dB of the right and left ear, respectively, requiring cochlear implants, and 1 infant had hearing thresholds of 60 and 40 dB of the right and left ear, respectively, requiring no hearing aids at 1.6 years of age. The worst outcome was seen in 16/26 symptomatic infants with severe cUS/MRI abnormalities (neuronal migration disorders only seen on MRI, cerebellar hypoplasia, ventriculomegaly, extensive periventricular calcifications, white matter cysts). In 1/16 infants with mild abnormalities on cUS (germinolytic cysts and LSV) occipital cysts as well as extensive polymicrogyria were noted with MRI (Figure 1). This infant is only 12 months old but has developed epilepsy and shows early signs of cerebral palsy. In 15/16 infants severe abnormalities were seen on cUS. In 12/15 (93%) infants a subsequent MRI showed severe (additional) cerebral abnormalities. Of these infants 3 died, neurodevelopmental sequelae and/or SNHL were seen in 8 survivors and in 1 survivor no signs of a delayed motor development but the first signs of cognitive delay were seen at the

Table 3. Neuro-imaging results and neurodevelopmental outcome of 33 infants.

Neuro-imaging abnormalities	No ND sequelae	Poor ND outcome	Cognitive impairment	Epilepsy	SNHL	Deceased
cUS						
No abnormalities (n=5)	5 (100)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Mild (n=16)[a]	15 (94)	1 (6)	0 (0)	1 (6)	2 (13)	0 (0)
Severe (n=15)[a]	0 (0)	8 (50)	8 (50)	4 (25)	7 (44)	6 (38)
MRI						
Mild (n=7)[a]	5 (100)	0 (0)	0 (0)	0 (0)	2 (17)	0 (0)
Severe (n=13)[a]	0 (0)	9 (69)	10 (77)	5 (38)	7 (54)	3 (23)

Data are presented as numbers with percentages in parentheses. [a] Infants could have more than one abnormality. Abbreviations: ND = neurodevelopmental; SNHL = sensorineural hearing loss.

age of 14 months. Of infants with neurodevelopmental sequelae, 6/8 have cerebral palsy and/or cognitive impairment with level V on the Gross Motor Function Classification System (age range: 1.6–18 years). Four of these infants developed postneonatal epilepsy requiring treatment with anti-epileptic medication; 2/8 infants had mild neurodevelopmental sequelae but no cerebral palsy.

Trimester of infection in relation to neuro-imaging and neurodevelopmental outcome

The trimester of infection was known in 17/36 (47%) infants. Ten infants (59%) were infected within the first, 3 (18%) during the second and 4 (23%) during the third trimester (Table 4).

First trimester

Eight out of 10 infants (80%) were symptomatic at birth (small for gestational age, microcephaly, convulsions, hepatitis, conjugated hyperbilirubinemia, thrombocytopenia). Seven of 8 symptomatic infants had severe cerebral abnormalities on cUS and MRI; 5 infants (63%) died; 2 (25%) had a poor neurodevelopmental outcome with cerebral palsy, cognitive impairment, epilepsy and bilateral SNHL for which they use hearing aids; 1 (12%) with mild cUS abnormalities did not show additional MRI lesions and had a normal neurodevelopmental out- come at the age of 1.4 years. The 2 asymptomatic infants were part of a quadruplet with variable expression of a first trimester congenital CMV infection as reported previously.[18] These infants had no or mild abnormalities on cUS and developed no sequelae and bilateral SNHL with mild cognitive delay, respectively, during the follow-up.

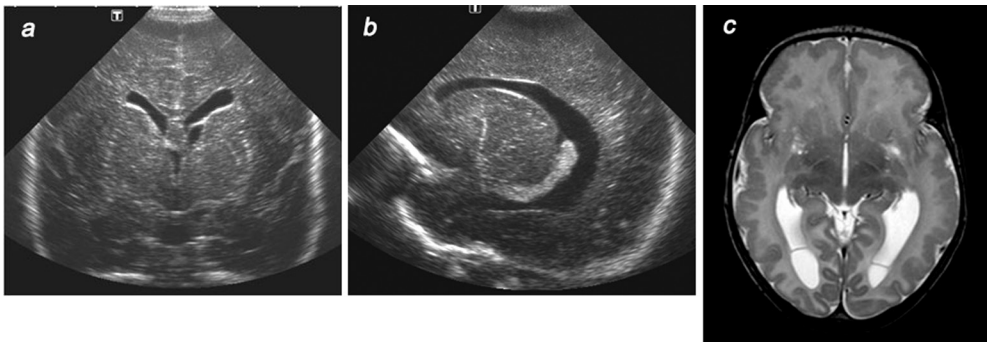

Figure 1. Infant born at term (39+3) with neuro-imaging findings suggestive of a first trimester infection. [A] cUS, coronal view showing a germinolytic cyst on the left and LSV bilaterally; [B] cUS, sagittal view showing LSV; [C] a T2-weighted MRI, axial view, additionally showing occipital cysts and extensive bilateral polymicrogyria.

Second trimester

One of 3 infants who was symptomatic at birth with mild cUS/MRI abnormalities has bilateral SNHL and has hearing aids at the age of 6.6 years; 2 of 3 infants were asymptomatic, had no and mild abnormalities on cUS, respectively, and have not developed sequelae at the age of 2 and 3.2 years.

Third trimester

Three out of 4 infants presented with thrombocytopenia and/or petechiae at birth. These infants had no or mild abnormalities on cUS/MRI, and none of them developed sequelae. One asymptomatic infant had no abnormalities on cUS. No MRI was performed, and outcome was normal at 1.2 years of age.

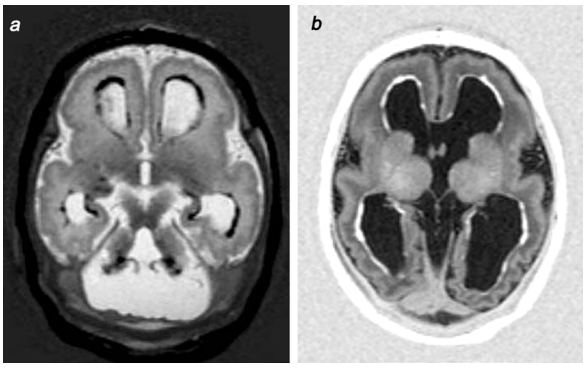

Figure 2. MRI, axial T1- [A] and T2- [B] weighted sequence showing ventricular dilatation and cerebellar hypoplasia. Periventricular calcification is seen as increased signal intensity on T1 and low signal intensity on the T2-weighted images. Extensive polymicrogyria is seen as well.

Table 4. Neuro-imaging results and neurodevelopmental outcome categorized in maternal trimester in which primary infection occurred.

	First trimester (n=10)	**Second trimester** (n=3)	**Third trimester** (n=4)	**Trimester unknown** (n=19)
Neuro-imaging				
MRI abnormalities				
Severe	4 (40)	0 (0)	0 (0)	9 (47)
Mild	2 (20)	1 (33)	2 (50)	2 (11)
None	0 (0)	0 (0)	0 (0)	0 (0)
No MRI	4 (40)	2 (67)	2 (50)	8 (42)
cUS abnormalities				
Severe	7 (70)	0 (0)	0 (0)	8 (42)
Mild	2 (20)	2 (67)	2 (50)	10 (53)
None	1 (10)	1 (33)	2 (50)	1 (5)
Outcome				
Mortality	5 (50)	0 (0)	0 (0)	1 (5)
SNHL > 30 dB				
Unilateral	0 (0)	0 (0)	0 (0)	0 (0)
Bilateral	5 (50)	1 (33)	0 (0)	4 (21)
ND impairment				
Severe	2 (20)	0 (0)	0 (0)	7 (37)
Mild	0 (0)	1 (33)	0 (0)	0 (0)
None	3 (30)	2 (67)	4 (100)	10 (53)
Unknown	5 (50)[a]	0 (0)	0 (0)	2 (11)
Epilepsy				
Yes	2 (20)	0 (0)	0 (0)	3 (16)
No	3 (30)	3 (100)	4 (100)	14 (74)
Unknown	5 (50)[a]	0 (0)	0 (0)	2 (10)[b]

Data are presented as numbers with percentages in parentheses. [a] Five patients were deceased before neurodevelopmental outcome could be assessed. [b] One patient was too young to assess neurodevelopmental outcome and one patient was deceased before neurodevelopmental outcome could be assessed. Abbreviations: ND = neurodevelopmental; SNHL = sensorineural hearing loss

Discussion

This study confirms previous findings that infants with cCMV infection acquired during the first trimester of pregnancy are at increased risk of symptomatic presentation with severe cerebral abnormalities, mortality and subsequent development of adverse sequelae such as cerebral palsy and SNHL.[19,20] In contrast, CMV acquired later in pregnancy is not associated with symptoms at birth or only with mild symptoms such as thrombocytopenia. These infants are likely to have no or mild cerebral abnormalities with subsequent normal neurodevelopmental outcome and hearing.

Transmission of CMV during the first trimester of pregnancy may interfere with early central nervous system development and brain maturation, and may cause severe cerebral abnormalities like disorders in neuronal migration and cerebellar hypoplasia. Presence of polymicrogyria has been related to an infection between 18 and 24 weeks of gestation.[8,12] Computed tomography has been recommended in the past as the gold standard to assess cerebral involvement in infants with cCMV infection, but recent studies have reported that MRI is more sensitive to show CMV-associated white matter and migrational abnormalities.[8,10,19,21-23] In our study cUS was superior in diagnosing LSV and germinolytic cysts. These cUS abnormalities suggest a cCMV infection. MRI provided additional information concerning neuronal migrational disorders. These severe abnormalities were invariably associated with a poor neurodevelopmental outcome and/or the development of SNHL. Bilateral generalized polymicrogyria has been related to cognitive and motor delay and epilepsy.[24] Without screening for primary cCMV infection during pregnancy, it is often unknown when the infection occurred. When only using cUS, the severity of cerebral abnormalities may be underestimated by not detecting disorders in neuronal migration, thus not allowing an accurate prediction of neurodevelopmental outcome.

The predictive value of abnormal white matter signal intensity on MRI, often seen in infants with cCMV, in relation to outcome has previously been studied.[10,25] In line with our findings, to date, no clear relation has been shown between these white matter lesions and neurodevelopmental outcome. A previous study from our group in infants with a postnatal CMV infection showed significantly reduced fractional anisotropy on diffusion tensor imaging in the occipital white matter, suggesting microstructural changes. These microstructural white matter changes did not appear to result in impaired neurodevelopmental outcome at 16 months' corrected age.[26]

In contrast to the literature, this study shows a high rate of symptomatic patients (72%).[1] This can be explained by the study design and the absence of systematic screening for cCMV in the Netherlands. Therefore, in general, only infants with a clinical suspicion of cCMV were referred to our hospital. Due to the retrospective design, a limitation of this study is the incomplete data regarding time of onset of infection and primary or reactivation of maternal CMV infection. In subsequent studies, these factors should be taken into account. Previously, primary maternal infections have been associated with more severe sequelae during follow-up.[27]

As the data were collected over more than 20 years, and as previous research did suggest that asymptomatic infants do not develop neurodevelopmental sequelae, MRI was not performed in those infants born during the early years of the study period.[4,28] As we have now shown (Figure 1, 2) that migrational disorders may even be present in infants with mild cUS findings, we have started and recommend to perform MRI in all infants with cCMV and cUS abnormalities.

In conclusion, infants with a first trimester cCMV infection are most at risk of severe cerebral abnormalities and neurological sequelae. MRI can provide additional information concerning migrational disorders and confirms findings seen on cUS, which can aid in the early prediction of outcome.

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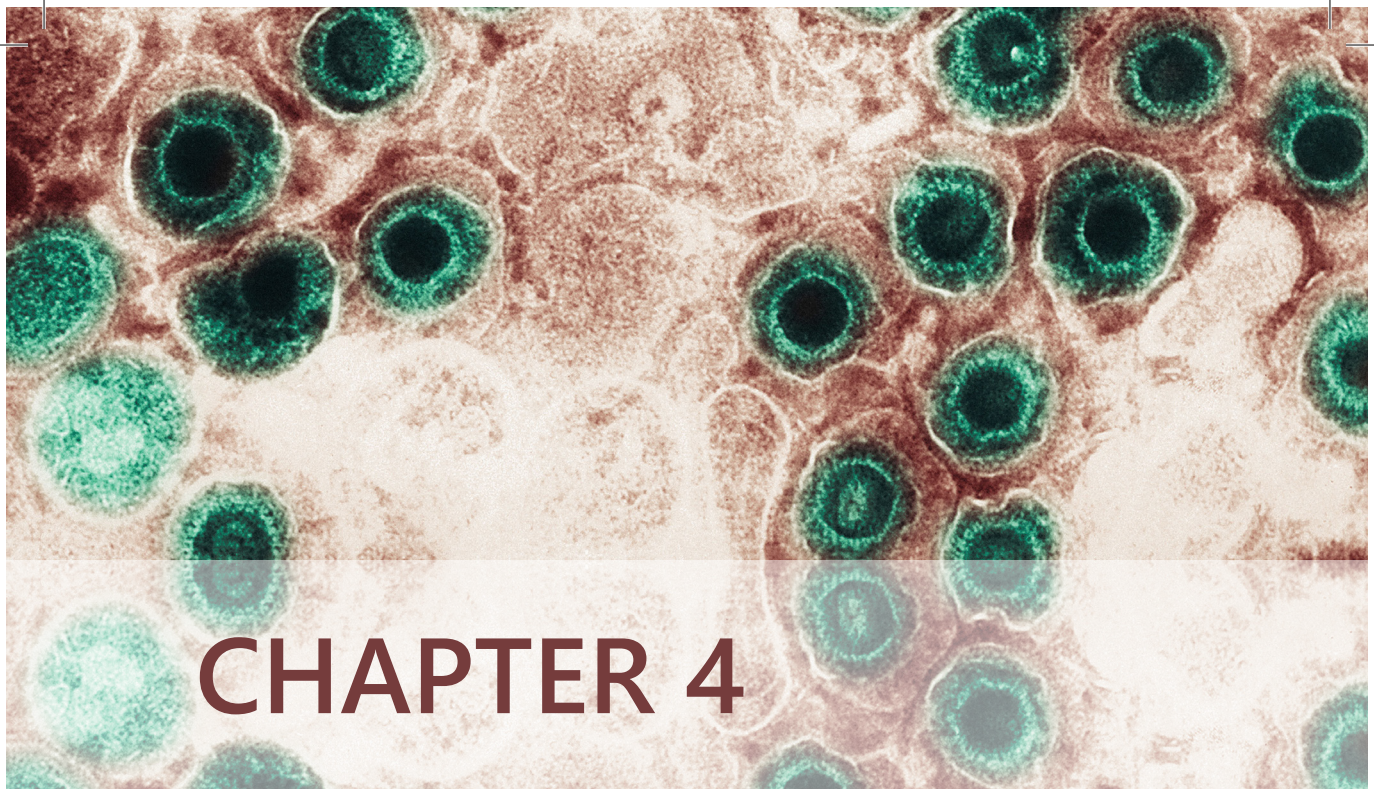

CHAPTER 4

Expert opinion and surveillance study on clinical symptoms, management and treatment of infants with congenital cytomegalovirus infection

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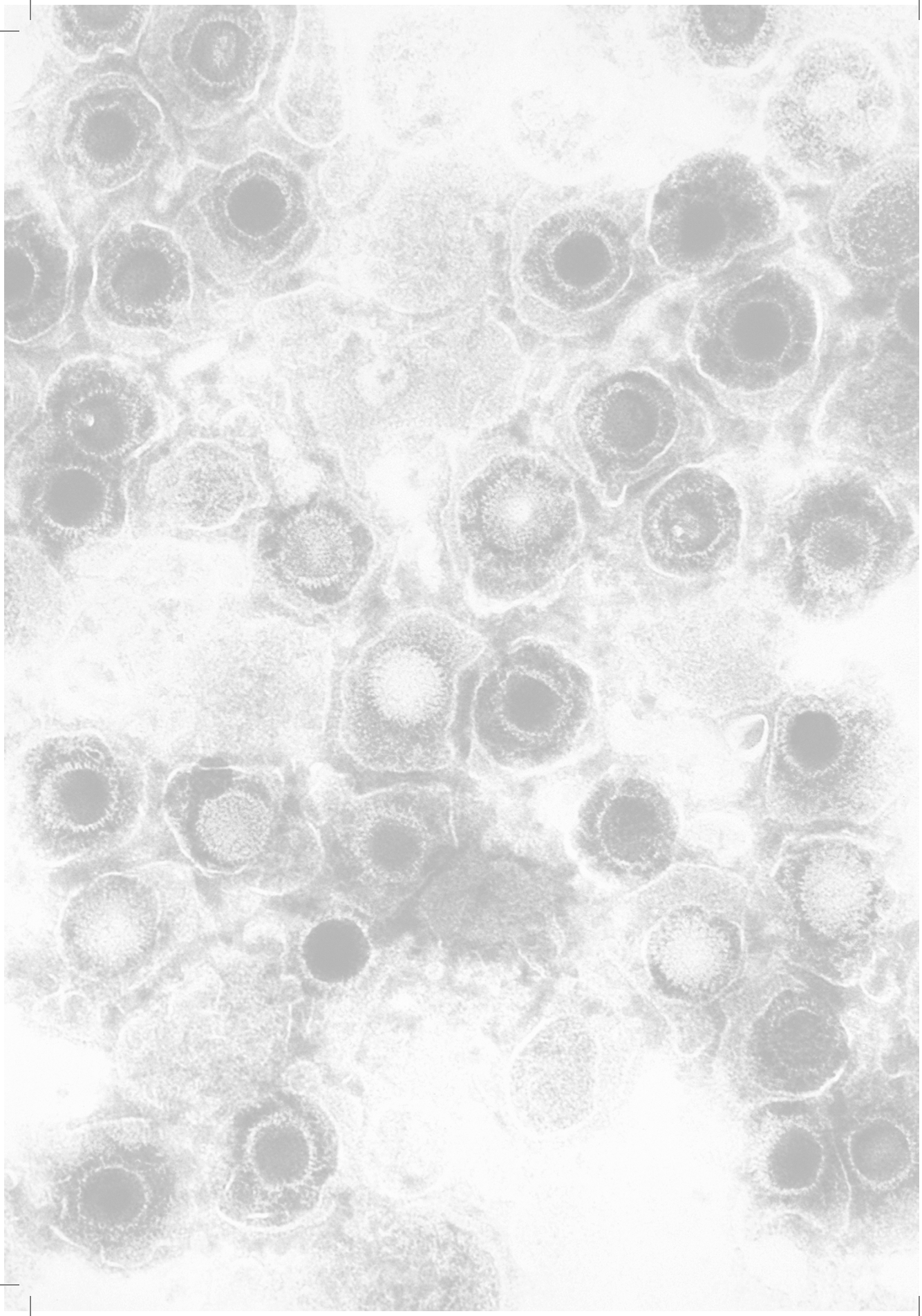

CHAPTER 4

Expert opinion and surveillance study on clinical symptoms, management and treatment of infants with congenital cytomegalovirus infection

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Abstract

Aim To evaluate the recognition and management practices of congenital cytomegalovirus (cCMV) infection among a select group of experts and through a national surveillance study.

Method From 2014 until 2015, a questionnaire was sent to experts involved in mother and infant care. Monthly surveillance was conducted among Dutch pediatricians for cases of cCMV infection from 2013 until 2015.

Results The questionnaire was completed by 63/103 (62%) respondents. The responses regarding recognition and management practices varied. Maternal screening was performed by 17/63 (27%) and infant screening by 3/61 (5%) of the respondents. Infant CMV diagnostics were most frequently initiated due to hepatosplenomegaly and, or, an increase in liver transaminases. Management practices included cranial ultrasound (57/63, 91%) and audiological follow up in symptomatic (61/63, 97%) and asymptomatic (52/63, 83%) infants. In terms of antiviral treatment, 46/63 (73%) treated symptomatic infants only, and 6/63 (9%) treated all infected infants. In total, 48 cases of cCMV infection were registered through the Dutch surveillance study, of which 43/48 (90%) infants were symptomatic.

Conclusion This study indicates that infants with cCMV infection are insufficiently recognized and highlights the need for consensus on management practices. Screening of infants and the development of an international management guideline are recommended.

Introduction

The human cytomegalovirus is the most common cause of fetal infection worldwide, with a global birth prevalence of 0.7%.[1] Congenital cytomegalovirus (cCMV) infection is the leading cause of non-genetic sensorineural hearing loss (SNHL) in developed countries.[2] Approximately 10-13% will be born symptomatic[1] and around 40-58% of these infants will develop permanent sequelae consisting of psychomotor, ocular and, or, auditory defects.[1] Among asymptomatic infants around 11.3% will develop late-onset SNHL.[2] In the Netherlands, the birth prevalence is estimated to be 0.5%, which culminates in approximately 1,000 children born with cCMV infection annually, of which roughly 130 are expected to be symptomatic at birth.[3] The burden of congenital CMV infections is considerable with annual estimates in the United States depicting CMV-associated long-term disabilities to occur more frequently than other common diseases such as Down's syndrome.[4] Significant advances have been made in the understanding of the epidemiology and management of cCMV infection.[5] Increased awareness of hygiene measures have shown to significantly reduce the risk of CMV transmission during pregnancy.[6] However, there are currently limited means to prevent CMV transmission and entirely effective treatment options are lacking. Imperative to the success of treatment and prevention options available[6,7] is awareness and knowledge of symptomatic cCMV presentation among healthcare providers, which is still very limited.[8,9] Even in the research setting, comparative studies on cCMV infection are hindered by varying definitions of symptomatology.[10] Accurate and timely identification of symptomatic infants would enable early intervention; still (universal) CMV screening is currently not advocated. In the absence of screening programs, registries have been set up in attempts to study the disease burden of cCMV infection.[11-13] Yet, low registration rates have been reported. The aim of this article was to determine the effectiveness of current practices in recognition and management of infants with cCMV infection among a selected group of experts using a questionnaire and among Dutch pediatricians using a national surveillance registry.

Method

Questionnaire study

An online questionnaire was developed covering seven cCMV related topics (Appendix S1) accompanied by a cover letter. The questionnaire was sent out between September 2014 and July 2015. It was distributed among a consortium of 49 neonatologists from 37 European neonatal centers with a focus on neonatal neurology. Hereafter, it was revised with four additional questions, marked with an asterisk in Appendix S1, and sent to medical contacts of the Effective Perinatal Intensive Care research group in Europe[14] (n=29) and 23 physicians involved in the European Congenital CMV Initiative who attended a meeting on cCMV infection at the European Society for Pediatric Infectious Diseases congress in Dublin, Ireland in May 2014. None of the existing questions were changed and there was no overlap between the

groups. Table S1 contains a detailed description of the distribution breakdown per country and per specialism. Participants were encouraged to forward the questionnaire to colleagues with an interest in the field. In total, the questionnaire was sent to 101 contacts.

Surveillance study

From January 2013 to December 2015 surveillance among pediatricians for cCMV infection was conducted for the first time in The Netherlands, through the Pediatric Association Of The Netherlands (NVK). The NVK operates a national surveillance system on several childhood conditions through the Dutch Pediatric Surveillance Unit. All active pediatricians in the Netherlands (n=1,453) received a monthly email reminder to register cases of cCMV online, after written parental consent was obtained. A case was included once cCMV infection was confirmed, parental consent was obtained and the intake form was completed. Two follow-up questionnaires were sent by the investigators at six months and 12 months regarding neurodevelopmental outcome including hearing and vision. A minimum of three reminders were sent out to non-responders, after which the child was marked as lost to follow up. Follow-up data was collected with the aim of studying disease burden and to relate symptoms at birth with outcome. All diagnostics were carried out as part of standard care. Ethical approval was waived as the surveillance study was part of a longstanding nationwide surveillance initiative (Nederlands Signalerings Centrum Kindergeneeskunde) of the NVK, which covers all hospitals in the Netherlands. This initiative was approved by the board of the NVK, encouraged by the Dutch Ministry of Health and strictly regulated by Dutch privacy laws. The study was conducted according to the national ethical guideline "Code Goed Gedrag", as published by the Dutch national association of medical scientific research, Federa.

Case definition and variables

Congenital CMV infection was defined as CMV detection by means of urine polymerase chain reaction (PCR), urine culture, saliva PCR and/or culture or serum PCR on samples taken within 21 days of age. For infants who were more than 21 days, CMV-PCR on dried blood spot cards taken within a few days after birth, were retrospectively analyzed as previously described[15] and included when positive. Infants with a postnatal CMV infection were excluded. Infants were considered symptomatic when one or more of the following symptoms were present: small for gestational age (birth weight <-2 standard deviations, SD), pneumonia, hepatomegaly, splenomegaly, jaundice, skin abnormalities (petechiae/ purpura), laboratory abnormalities (thrombocytopenia, elevated liver transaminases, hyperbilirubinemia) and/or central nervous system (CNS) involvement. CNS involvement was defined as microcephaly (head circumference <-2 SD), intracranial calcifications, hydrocephalus, germinolytic cysts, white matter cysts, occipital cysts, polymicrogyria, cerebellar hypoplasia, abnormal cerebrospinal indexes or detection of CMV-DNA in cerebrospinal fluid, chorioretinits and hearing deficits.[5,7,16]

Data management

The data was managed and analyzed using IBM SPSS Statistics (version 23.0, IBM Corp. ©, New York, USA). Data were summarized according to frequency (mean, range and percentage).

Results

Questionnaire study

A total of 63/101 (62%) returned the questionnaire. The respondents were from 19 countries in Europe and one response was from South Africa (Table S1). The respondents were all physicians and the majority were neonatologists (40/63; 63%) followed by pediatric infectious disease experts (10/63, 16%), obstetricians (8/63, 13%), general pediatricians (4/63, 6%) and one (2%) was unspecified.

Screening

Screening during pregnancy was conducted by 17 (27%) respondents, 43 (68%) would only screen upon indication, two (3%) were not sure and one participant (2%) left the question unanswered. Of the 17 participants that answered yes, five (29%) screened in the first trimester, four (24%) every trimester until seroconversion, two (12%) in the first and second trimester, one (6%) in the first and third trimester and five (29%) left this question unanswered. There was considerable variation in response to this question between countries and within countries (Figure S1). CMV-hyperimmune globulins were always administered after seroconversion by six (9%) of the respondents, 17 (27%) respondents answered never, three (5%) only when fetal ultrasound abnormalities were present and eight (13%) were not sure. Twenty-nine (46%) respondents left this question unanswered. Among the respondents, 34 (54%) considered it important to screen for CMV during pregnancy, 27 (43%) did not think so and two (3%) gave no answer. Only three (5%) respondents indicated that infants were screened at their institution, 56 (89%) indicated that infants were only tested for CMV upon clinical indication and four (6%) left this question unanswered (Figure S1.1).

Infant symptomatology and diagnostics

Hepatosplenomegaly or increase in liver transaminases was most frequently chosen by 55 (87%) of the respondents, followed by hearing loss in 53 (84%) and thrombocytopenia in 52 (83%) (Figure 1A). Other symptoms that would suggest cCMV infection not included in the questionnaire, as proposed by some of the respondents were prematurity, migrational disorders and white matter damage, microcephaly, inguinal hernia and maternal CMV-like illness. One respondent indicated they would choose all symptoms if there was no alternative explanation. All nine symptoms were chosen by 22 (35%) of the respondents (Figure S2). The most common material used to test for CMV was urine, followed by blood (Figure 1B).

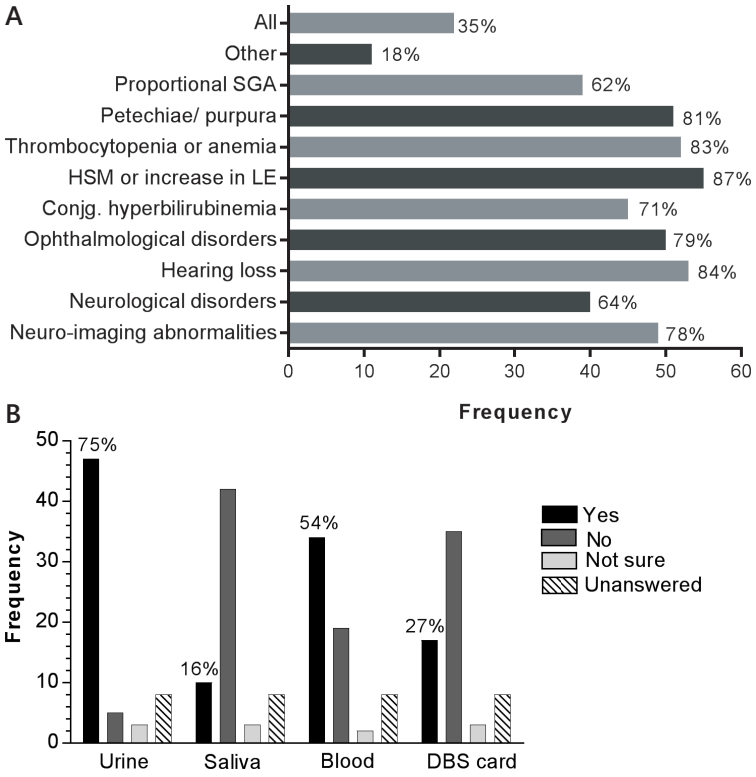

Figure 1. [A] Chosen symptoms that would prompt CMV diagnostics in the infant and [B] most frequently used materials to test for CMV. SGA: small for gestational age, HSM: hepatosplenomegaly, LE: liver enzymes, DBS: dried blood spot.

4

Infant neuro-imaging

A standard cranial ultrasound (cUS) was performed by 57 (91%) of the respondents, two (3%) did not perform standard cUS, two (3%) performed standard cUS only in symptomatic infants, and two (3%) left this question unanswered. The two respondents, who did not perform a standard cUS, both performed a standard cranial magnetic resonance imaging (MRI) instead. A standard cranial MRI was performed during the initial admission period by 28 (44%) respondents, 17 (27%) performed MRI only after structural abnormalities were seen on cUS, 10 (16%) performed no standard MRI, six (10%) were not sure and two (3%) left the question unanswered (Figure S3). The majority (86%) indicated that cranial computed tomography (CT) scans were not performed during the initial admission period; however one (2%) respondent performed CT scans as part of standard care. A CT scan was performed only when abnormalities were seen on cUS by two (3%) respondents; four (6%) were unsure and two (3%) left this question unanswered. Lastly, 43 (68%) respondents thought that neuro-imaging was very important in the management of both symptomatic and

asymptomatic infants, 17 (27%) thought that it was moderately important and only in symptomatic infants and three (5%) did not give an indication.

Antiviral treatment
Treatment of exclusively symptomatic infants with antiviral agents (ganciclovir/valganciclovir) was done by 46 (73%) respondents at their hospital, six (9%) treated both symptomatic and asymptomatic infants, three (5%) did not treat with antivirals at all, three (5%) respondents were not sure and two (3%) did not give a response. Other treatment indications presented by three (5%) respondents included treatment of infants with CNS involvement or on life-threatening indication only, symptomatic infants and infants that are included in a national study only, and only at recommendation of the pediatric infectious disease expert. Treatment duration varied considerably but was mostly six weeks chosen by 31 (52%) respondents followed by six months chosen by eight (13%) respondents, 12 months in one case (2%), 10 (17%) respondents were not sure and three (5%) gave no answer. Other answers given by, seven (11%) respondents were that duration was dependent on the severity of the symptoms or the advice of the clinical virologist.

Hearing and ophthalmological examination
Audiological follow up in symptomatic infants was performed by 61 (97%) respondents and two (3%) left this question unanswered (Figure S4). Audiological follow up in asymptomatic infants was done by 52 (83%) respondents, four (6%) did not, four (6%) were not sure and three (5%) left this question unanswered. There was great inconsistency about the timing of audiological follow up. The majority, (32%) indicated during admission, at three months, 12 months and yearly until six years of age. Ophthalmological examination before discharge was done in symptomatic infants only among three (10%) of the respondents while among 22 (71%) respondents this was done in both symptomatic and asymptomatic infants, four (13%) were not sure and one (6%) gave no response (Figure S4.1). Follow-up examinations were carried out in symptomatic infants only among two (6%) respondents, while nine (29%) respondents did this among infants with pre-existing eye abnormalities only, and another nine (29%) (n=9) among both symptomatic and asymptomatic infants regardless of the result of the initial examination. Eight (26%) respondents were not sure about ophthalmological follow up, two (6%) gave no answer and one (3%) indicated that the need for follow up was determined per patient by the ophthalmologist.

Neurodevelopmental follow up
Neurodevelopmental follow up was performed according to 52 (82%) respondents; five (8%) indicated that infants were only seen upon clinical indication, one (2%) indicated that infants were not seen for follow up at all, three (5%) were not sure, and two (3%) left this question unanswered (Figure S5). The most frequently used assessment tool was the Bayley Scales of Infant and Toddler Development III (45%); however, there was great inconsistency (Figure S5.1).

Surveillance study

During the study period, 55 registrations were made of which 48 (87%) could be used (n=1 no parental consent, n=1 lost to follow up; n=2 incorrect registrations, n=3 postnatal CMV). The majority of infants were symptomatic at birth (90%) (Table 1).

All cases were diagnosed due to symptoms in the neonatal period or antenatal suspicion. The most frequent symptoms were neuro-imaging abnormalities as seen on any of the three modalities (65%), followed by hearing loss (46%) and thrombocytopenia (45%). In total, three (6%) infants died within one week after birth due to complications of fulminant CMV-disease. Of the infants that died, one was full-term (gestational age 37 weeks + 6 days) and the other two were preterm infants (gestational age 30 weeks and gestational age 29 weeks + 6 days). Only one of the three infants that died received antiviral treatment for one day before clinical deterioration. Among the rest of the infants, 23 (48%) were treated with (val)ganciclovir for mostly six weeks (78%) however, three (13%) infants were treated for six months. Neonatal hearing loss was present in 22 (46%) infants, of whom 12 (55%) received antiviral treatment. Of the treated group with hearing loss (n=12); the hearing outcome at six months was known for 10/12 (83%) children and all still had hearing loss (five stable; three progressive), except in two the outcome was inconclusive. At 12 months, the hearing outcome for this group was known for 8/12 (67%) children of whom six were stable, one improved but still had hearing loss and one child with unilateral neonatal hearing loss now had bilateral hearing loss. At six months, follow-up information was known for 39/48 (81%) children (n=1 diagnosis of cCMV came after six months of age, n=4 lost to follow up, n=1 not six months old yet during analysis, n=3 deceased).

Developmental abnormalities were seen in 13/39 (33%) children and included delays in neurological and motor milestones, seizures, delayed/no language development, hyper- and hypotonia. All 13 children were symptomatic at birth and all but one had CNS involvement. Antiviral therapy in the neonatal period was administered to 7/13 (54%) children with abnormal development at six months and 13/25 (52%) children with normal development. At 12 months, follow-up information was known for 34/48 (71%) children (n=4 lost to follow up, n=7 not 12 months old yet during analysis, n=3 deceased) of whom, 7/34 (21%) had developmental disabilities such as delayed speech development and delayed psycho-motor development. All children also had developmental delays at six months.

Antiviral therapy in the neonatal period was administered to 5/7 (71%) children with abnormal development at 12 months of age and 12/27 (44%) children with normal development.

Table 1. Neonatal characteristics of 48 cases of congenital CMV infection in The Netherlands.

Infant characteristics	
Gestational age, mean, weeks (range)	37 (27–41)
Preterm birth (GA < 37 weeks)	13 (27)
Place of birth	
Hospital	45 (94)
Home	3 (6)
Symptomatic cCMV infection	43 (90)
Asymptomatic cCMV infection	5 (10)
CNS-involvement	37 (77)
Non-CNS abnormalities	
Petechiae/ purpura	10 (21)
Small for gestational age	7 (15)
Jaundice	4 (8)
Hepatosplenomegaly	3 (6)
Pneumonia	1 (2)
Other (viral cardiomyopathy)	1 (2)
Laboratory abnormalities*	
Thrombocytopenia (n=38)	17 (45)
Anaemia (n=40)	12 (30)
Hyperbilirubinemia (n=36)	6 (17)
Elevated liver transaminases (n=36)	8 (22)
CNS abnormalities	
Microcephaly	9 (19)
Intracranial calcifications (n=45)	22 (49)
Abnormal cerebrospinal fluid for age (n=2)	0
Chorioretinits (n=45)	2 (4)
Hearing deficits	22 (46)
Seizures	2 (4)
Apnoea	2 (4)
Hypotonia	2 (4)
Neuro-imaging abnormalities; total (n=45)	31 (65)
Abnormal cUS (n=45)	30 (67)
Abnormal MRI (n=25)	18 (72)
Abnormal CT	0
Antiviral treatment	23 (48)
Neonatal death	3 (6)
Neurodevelopmental outcome	
Abnormal at 6 months (n=39)	13 (33)
Abnormal at 12 months (n=34)	7 (21)

Data are presented as numbers with percentages or mean/medians with ranges in parentheses.
* Thrombocytopenia: <150x10^9/L; Anaemia: <8 mmol/L; Hyperbilirubinemia: total:>140 umol/L, direct >10 umol/L; Hepatitis: transaminases >2x normal. Abbreviations: CNS: central nervous system, cUS: cranial ultrasound, MRI: magnetic resonance imaging, CT: computed tomography.

Discussion

Congenital cytomegalovirus infection is a public health issue of major concern.[4] There is no global consensus on disease characteristics or management of cCMV infections. Different definitions of symptomatology,[10] insufficient knowledge among healthcare providers[8] and low registration rates in cCMV registries indicate a variable state of current cCMV standard practices. Using a questionnaire we aimed to get an indication of how efficiently cCMV infected infants are recognized and clinically managed among a select group of experts. Furthermore, we conducted a surveillance study to investigate the effectiveness of these practices among Dutch pediatricians. The results of the questionnaire study indicated that there is little consensus in the recognition and management of infants with cCMV infection. Even though maternal and infant screening are currently not recommended,[17] 17 respondents (27%) indicated that pregnant women were screened and three (5%) indicated that infants were screened at their institution. The importance of early identification is that it may aid in detecting hearing impairments at an early stage through periodic audiological follow up. Rapid intervention has shown to significantly improve speech- and language development in the first years of life, even in infants with unilateral hearing loss.[18-20]

To further examine the success of infant recognition, participants were presented a list of characteristic cCMV symptoms asking which ones would trigger CMV diagnostics. Merely 35% of the respondents chose all symptoms. Hearing loss, the hallmark of cCMV infections was chosen by 53 (84%) respondents only, indicating that despite this characteristic symptom, CMV is still not recognized. In terms of management practices, reassuringly, the majority of the respondents indicated that a standard cUS was performed in all infected infants. A small fraction indicated that CT scans were carried out, however CT scans are inferior to MRI and do not provide additional information to cUS.[22] Inconsistencies in management practices were noted with regard to antiviral treatment and follow up. The majority indicated that only symptomatic infants were treated; however several respondents also indicated treating asymptomatic infants despite lack of efficacy data in this population. Audiological follow up in symptomatic infants was conducted among 97% of the respondents; however, no uniformity was seen when asked about the timing (data not shown). Only 83% indicated that asymptomatic infants had audiological follow up and 6% indicated no audiological follow up for both groups. This is of major concern as audiological follow up should be 100% in view of late onset and progressive hearing loss.[1,2]

In the national surveillance study, merely 55 infants were reported over three years, of which 48 could be included. With around 515,000 infants born in The Netherlands over the study period[23] and a birth prevalence of 0.5%[3] of which 13% will be symptomatic, we would have expected roughly 360 symptomatic cases in three years. This indicates that about 80-90% of symptomatic infants may not have been reported. The reason for this low response rate could be due to under-reporting. On the other hand it is conceivable that missed-diagnoses due to the often non-specific nature of the symptoms may be an important reason, a point that has been previously repor-

ted.[11,13,24] Universal screening could circumvent these problems. The generated data from screening may also aid in characterizing disease burden and creating a uniform and evidence based approach to clinical management. CMV infection was fatal for 6% of the infants in the surveillance study. Mortality rates in the literature may vary in the range of 10-30% among symptomatic infants[1] but may be as low as 4%.[24] Administration of antivirals did not seem to improve hearing in this small group of infants with hearing loss, until 12 months of age. No conclusions on antiviral efficacy should be drawn from this however, due to the small number of included infants. Due to the low registration rate it was not possible to study disease burden or correlate symptoms at birth to outcome.

There are several limitations to this study. The survey participants were a selected sample and therefore the conclusions cannot be generalized. Despite selective sampling among a group of experts, it is likely that random sampling of all pediatricians may reveal even less favorable results. Furthermore, the results may not be seen as general representations of different countries. Due to the low inclusion number in the surveillance study, the findings cannot be generalized to the Netherlands as selection- and referral bias led to an unrepresentative selection of symptomatic infants. Moreover, CMV diagnostic methods included serum sampling, which may have been a risk for false negative results due to the lower sensitivity of serum compared to urine or saliva.[25]

Conclusion

In conclusion, there was limited consensus regarding identification and management of infants with cCMV infection among expert healthcare professionals. Universal screening of cCMV infection may improve identification of all infected infants and detect infants benefiting from early intervention. The public health concern should fuel research on national or localised universal screening programs to explore its feasibility. Data from large screening programs may promote development of international guidelines regarding uniform, evidence based recommendations in the care of infants with cCMV infection.

Supplemental data

S1 – Questionnaire (*Additional questions added after revision)

1. Name
2. City, Country
3. Email address
4. Occupation
5. Which of the following signs would urge you to test for a congenital CMV infection? (You may tick multiple boxes)
   - □ Proportional small for gestational age infant
   - □ Petechiae or purpura
   - □ Thrombocytopenia or anemia
   - □ Hepatosplenomegaly or increase in liver transaminases
   - □ Conjugated hyperbilirubinemia
   - □ Ophthalmological disorders: chorioretinits, retinal bleeding
   - □ Hearing loss
   - □ Neurological disorders: lethargy, hypotonia, convulsions, elevated protein in cerebrospinal fluid
   - □ Abnormalities on cranial neuro-imaging: germinolytic cysts, lenticulostriate vasculopathy, ventriculomegaly
   - □ Other: ...

Screening - pregnancy

1. Are pregnant women screened for CMV serostatus during pregnancy?
   - □ Yes
   - □ No, only upon indication (go to question 1.3)
   - □ Not sure

1.1 If yes, when are pregnant women screened during pregnancy? You may tick multiple boxes
   - □ First trimester
   - □ Second trimester
   - □ Third trimester
   - □ Every month (until seroconversion)
   - □ Not sure
   - □ Never
   - □ Other: ...

1.2 Are CMV hyperimmune globulins administered after seroconversion?
   - □ Yes, always
   - □ Yes, only when fetal ultrasound abnormalities are present
   - □ No
   - □ Not sure
   - □ Other: ...

1.3 Do you think it is important to screen for congenital CMV infection? (You may tick multiple boxes)
   - □ Yes, prior to pregnancy
   - □ Yes, during pregnancy
   - □ Yes, after birth
   - □ Yes, during pregnancy and after birth
   - □ No, only upon clinical indication
   - □ Other: ...

S1 – Questionnaire — continued

Screening – infant

2. Are infants screened for congenital CMV infection at your hospital/institution?
   - □ Yes
   - □ No, only upon indication
   - □ Not sure

2.1 With what material are infants screened? (You may tick multiple boxes)
   - □ Urine
   - □ Saliva
   - □ Blood
   - □ Dried blood spot cards
   - □ Not sure
   - □ Other: ...

Neuro-imaging

3. Do you think that infants with a congenital CMV infection should get a standard cranial ultrasound?
   - □ Yes
   - □ No
   - □ Not sure
4. Do you think that infants with a congenital CMV infection should get a standard CT scan?
   - □ Yes
   - □ No
   - □ Not sure
5. Do you think that infants with a congenital CMV infection should get a standard MRI scan?
   - □ Yes
   - □ No
   - □ Not sure
6. Once a congenital CMV infection is diagnosed, are infants examined with cranial ultrasound for structural brain abnormalities?
   - □ Yes, but only symptomatic infants
   - □ Yes, symptomatic and asymptomatic infants, as part of standard care
   - □ No
   - □ Not sure
7. Do infants with a proven congenital CMV infection get a MRI during the initial admission period?
   - □ Yes, only when structural abnormalities are present on cranial ultrasound
   - □ Yes, as part of standard care
   - □ No (go to question 8)
   - □ Not sure

7.1 If yes, at what postnatal age do infants with a congenital CMV infection get a MRI scan? (You may tick multiple boxes)
   - □ Always during the first admission
   - □ After discharge
   - □ At term equivalent age
   - □ Not sure
   - □ Other: ...

S1 – Questionnaire — continued

8. Do infants with a proven congenital CMV infection get a standard CT scan during the initial admission period?
   - □ Yes, only when structural abnormalities are present on cranial ultrasound
   - □ Yes, as part of standard care
   - □ No (go to question 9)
   - □ Not sure

8.1 If yes, at what postnatal age do infants with a congenital CMV infection get a CT scan? (You may tick multiple boxes)
   - □ Always during the first admission
   - □ After discharge
   - □ At term equivalent age
   - □ Not sure
   - □ Other: ...

9. How important do you think neuro-imaging is in the care of infants with a congenital CMV infection?
   - □ 0 – not important
   - □ 1 – moderate, only in symptomatic infants
   - □ 3 – very, in both symptomatic and asymptomatic infants

Hearing test

10. Are symptomatic infants followed up audiologically?
   - □ Yes
   - □ No (go to question 11)
   - □ Not sure

10.1 When are symptomatic infants followed up audiologically?
   - □ During the first admission
   - □ At 3 months
   - □ At 6 months
   - □ At 12 months
   - □ Yearly until 6 years of age
   - □ Never
   - □ Not sure
   - □ Other: ...

11. Are asymptomatic infants followed up audiologically?
   - □ Yes
   - □ No
   - □ Not sure

11.1 When are asymptomatic infants followed up audiologically? (You may tick multiple boxes)
   - □ During the first admission
   - □ At 3 months
   - □ At 6 months
   - □ At 12 months
   - □ Yearly until 6 years of age
   - □ Never
   - □ Not sure
   - □ Other: ...

S1 – Questionnaire — continued

Ophthalmology test

12. Are infants with a proven congenital CMV infection seen by an ophthalmologist before discharge/ in the neonatal period?*
    - □ Yes, only symptomatic infants
    - □ Yes, both symptomatic and asymptomatic infants as part of standard care
    - □ No (go to question 13)
    - □ Not sure
    - □ Other: ...

12.1 Are routine ophthalmology checks after discharge carried out in infants with a congenital CMV infection?*
    - □ Yes, only symptomatic infants
    - □ Yes, only if eye abnormalities are detected in the neonatal period
    - □ Yes, in both symptomatic and asymptomatic infants as part of standard care
    - □ No
    - □ Not sure
    - □ Other: ...

Follow-up

13. Are infants with congenital CMV seen in the follow-up clinic?
    - □ Yes
    - □ Only upon clinical indication
    - □ Not sure
    - □ No

13.1 Which neurodevelopmental assessment tool is used in the follow-up clinic? (You may tick multiple boxes)*
    - □ Griffiths Mental Development Scales
    - □ Bayley Scales of Infant and Toddler Development II
    - □ Bayley Scales of Infant and Toddler Development III
    - □ Movement Assessment Battery for Children
    - □ Kaufmann Assessment Battery for Children
    - □ Wechsler Preschool and Primary Scales of Intelligence
    - □ Denver Developmental Screening Test
    - □ Not sure
    - □ Other: ...

Treatment

14. Do you use antiviral agents (ganciclovir/ valganciclovir) for the treatment of congenital CMV infection at your institution/ hospital?
    - □ Yes, only in symptomatic infants
    - □ Yes, in both symptomatic and asymptomatic infants
    - □ No
    - □ Not sure
    - □ Other: ...

14.1 If yes, what is the average duration of antiviral treatment? (Total treatment period)
    - □ 6 weeks
    - □ 6 months
    - □ 12 months
    - □ >12 months
    - □ Not sure
    - □ Other: ...

Table S1. Distribution of questionnaire sent per country and per specialism and total responses. Note: participants were asked to forward the questionnaire, hence some countries were initially not included in the 'total sent' however, responses were received (i.e. Iceland).

Countries sent	Total sent	Total returned
UK	35	9
NL	23	11
Greece	3	2
Belgium	10	3
Italy	21	6
Germany	11	4
Spain	2	3
Estonia	2	2
Poland	3	1
Portugal	3	1
Sweden	13	6
Denmark	6	5
France	7	2
Switzerland	4	2
Norway	2	1
Slovenia	1	1
Ireland	1	0
Finland	1	0
South Africa	0	1
Austria	0	1
Iceland	0	2

Per profession sent	Total sent	Total returned
Neonatologist	69	40
Pediatrician	11	4
Pediatric infectious disease expert	13	10
Obstetrician	2	8
Virologist	3	0
Clinical pediatric researcher	3	0
Unspecified	0	1

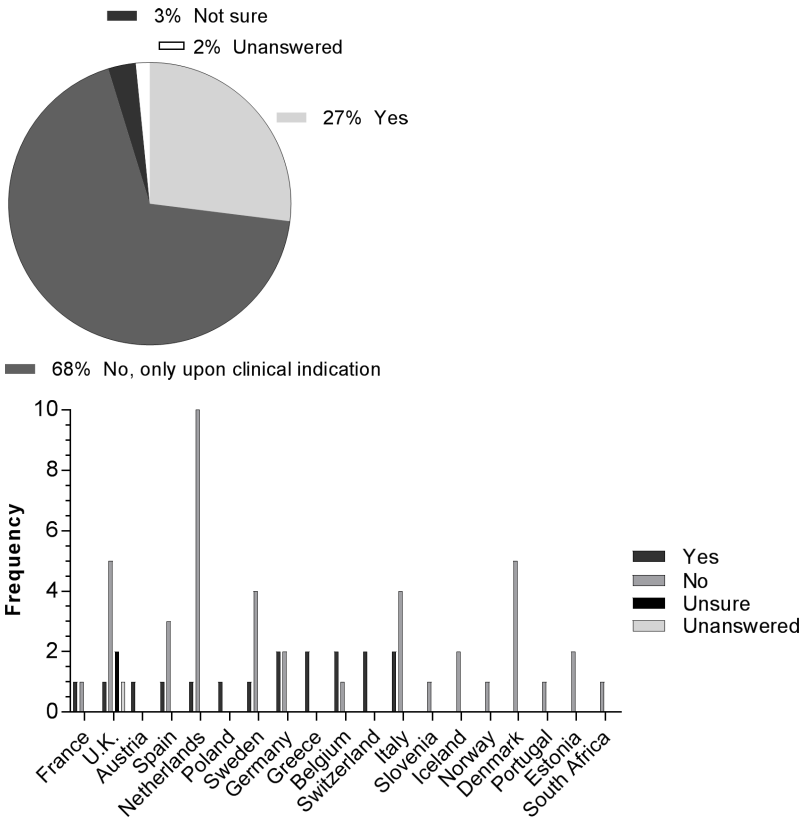

Figure S1. Screening. Are pregnant women screened for CMV-serostatus during pregnancy? The second figure represents the responses per country.

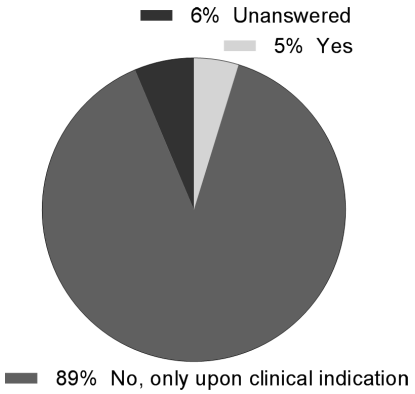

Figure S1.1. Are infants screened for congenital CMV infection at your hospital/institution?

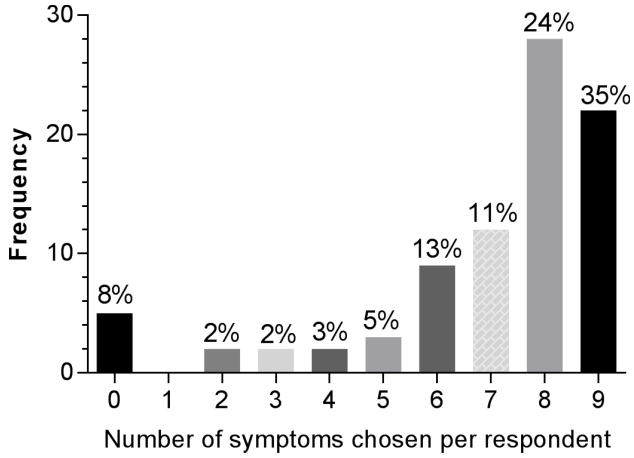

Figure S2. Infant symptomatology; total number of clinical symptoms chosen out of nine options.

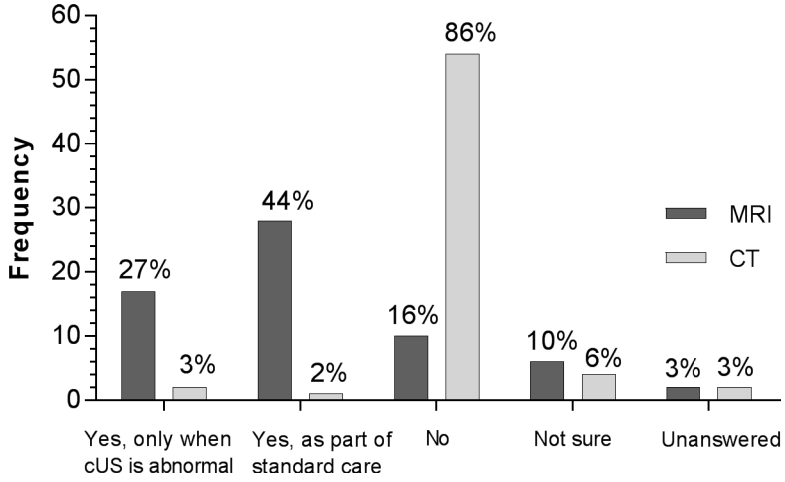

Figure S3. Neuro-imaging. Do infants with a proven congenital CMV infection get a standard MRI/ CT scan during the initial admission period?

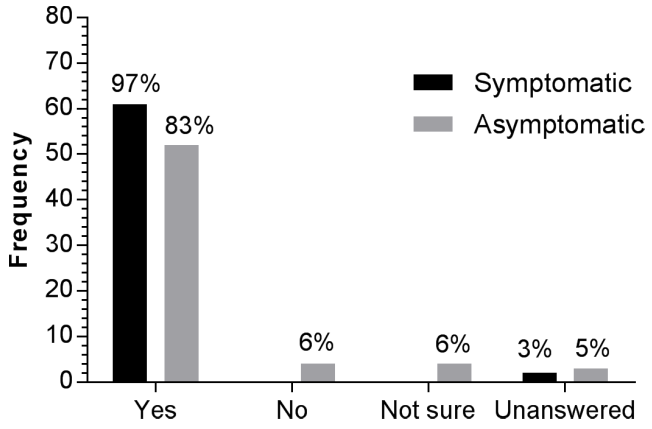

Figure S4. Hearing and ophthalmology. Are (a)symptomatic infants followed up audiologically?

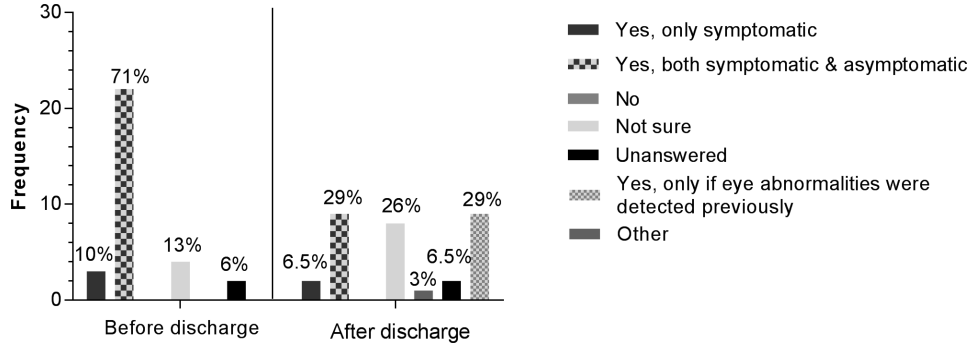

Figure S4.1. Are infants with a proven congenital CMV infection seen by an ophthalmologist before discharge/in the neonatal period and after discharge?
(Combined results for question 12 and 12.1)

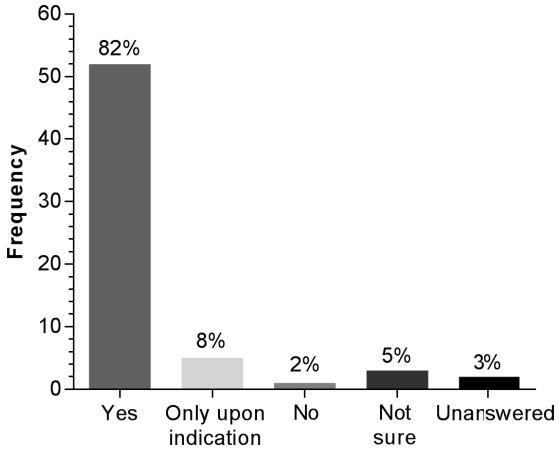

Figure S5. Follow-up. Are infants with congenital CMV seen in the follow-up clinic?

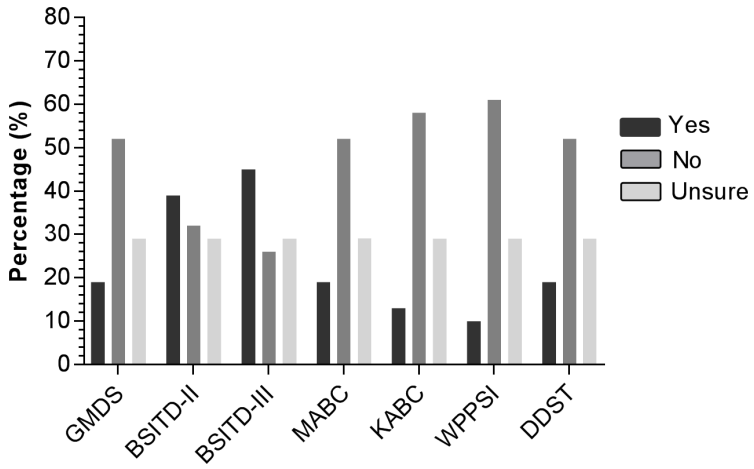

Figure S5.1. Which neurodevelopmental assessment tool is used in the follow-up clinic?

Abbreviations
GMDS: Griffiths Mental Development Scales
BSITD-II: Bayley Scales of Infant and Toddler Development II
BSITD-III: Bayley Scales of Infant and Toddler Development III
MABC: Movement Assessment Battery for Children
KABC: Kaufmann Assessment Battery for Children
WPPSI: Wechsler Preschool and Primary Scales of Intelligence
DDST: Denver Developmental Screening Test

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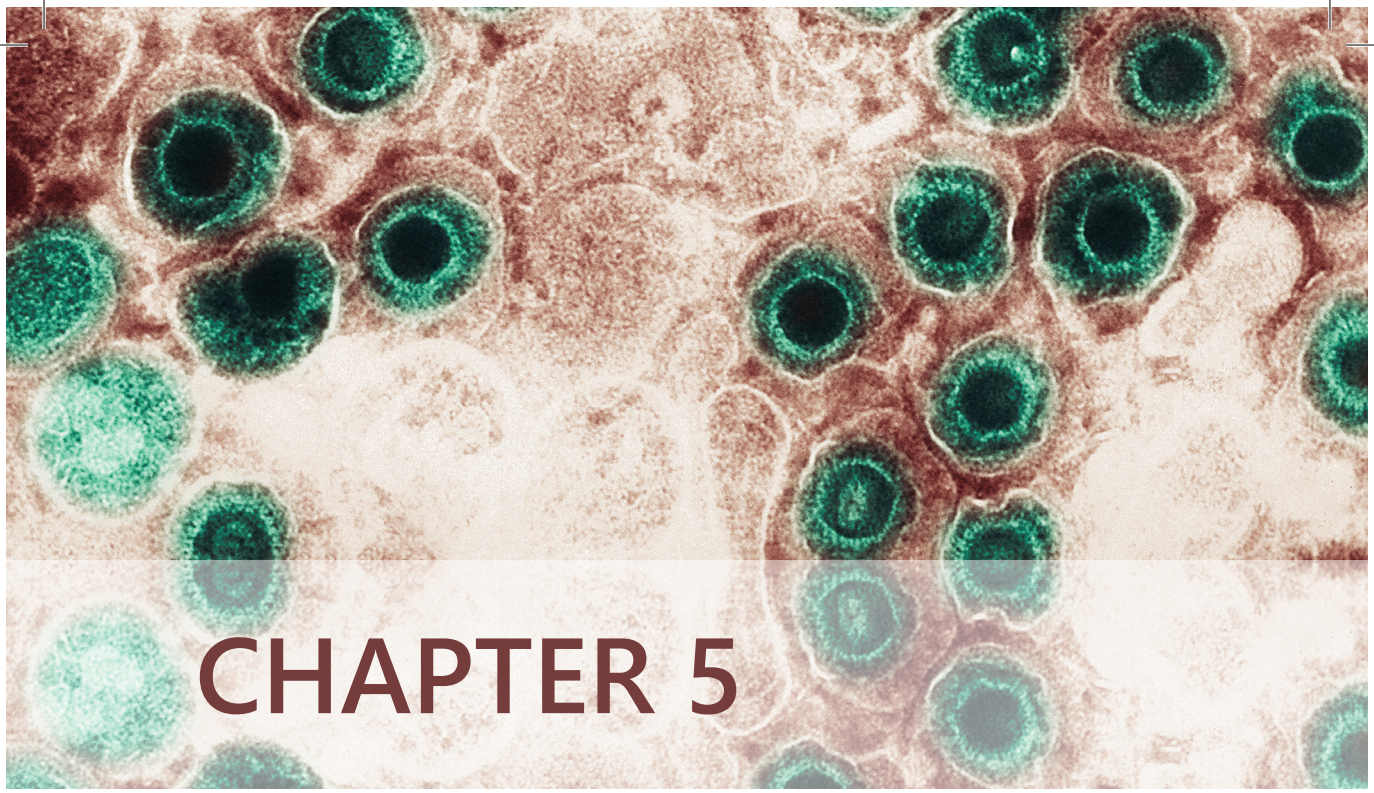

CHAPTER 5

Predictors of severity for postnatal cytomegalovirus infection in preterm infants and implications for treatment

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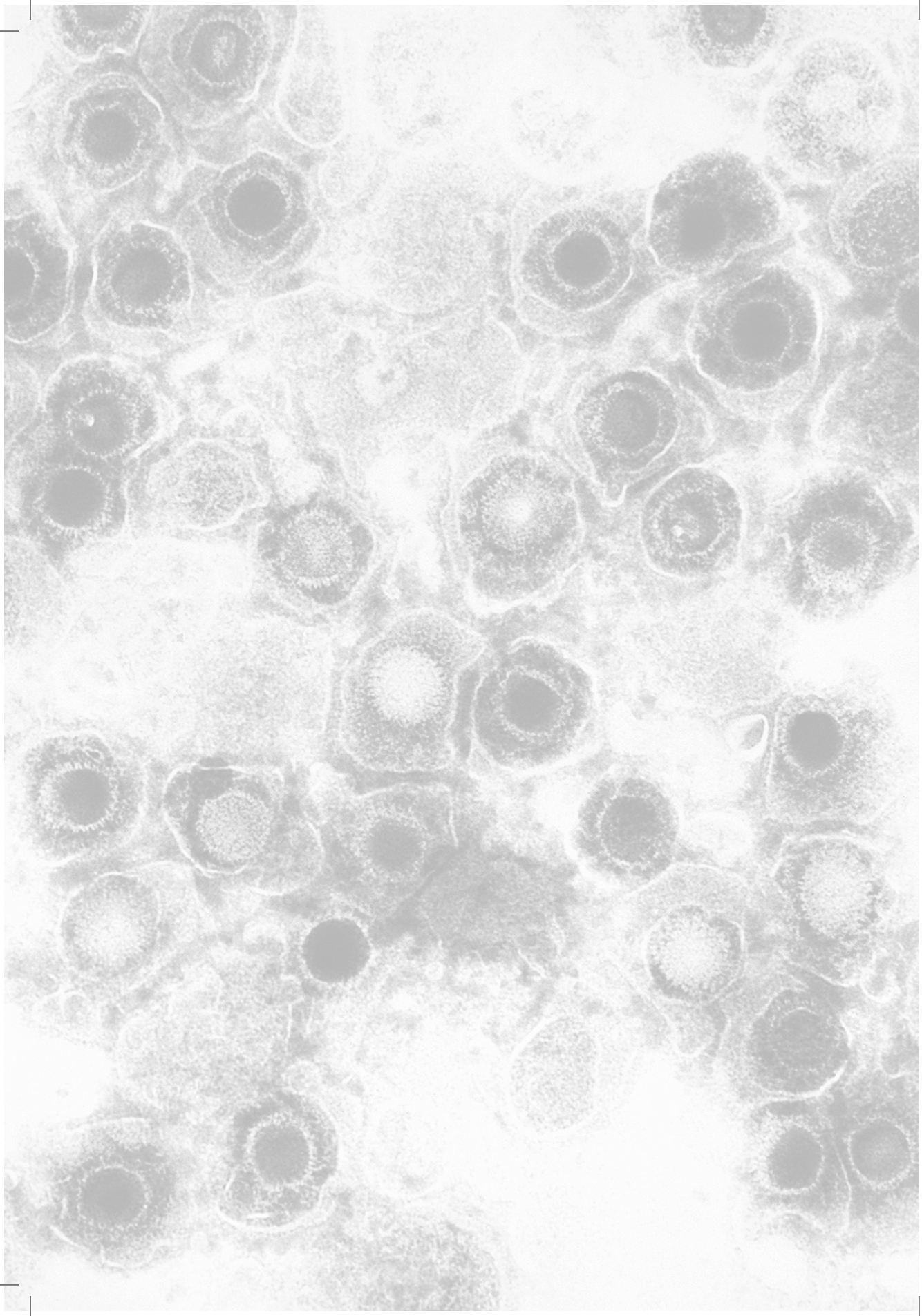
Linda S. de Vries
Joppe Nijman



CHAPTER 5

Predictors of severity for postnatal cytomegalovirus infection in preterm infants and implications for treatment

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Abstract

Postnatal cytomegalovirus (CMV) infection is common in neonates and is mostly acquired through infected breast milk from seropositive mothers. In this review, risk factors of postnatal CMV transmission and predictors of severity, preventive measures and treatment of symptomatic postnatal CMV infection in preterm infants are discussed. Several viral, transmission route and host factors have been associated with a higher risk of postnatal CMV transmission from mother to child. Severity predictors of symptomatic postnatal CMV infection may include extreme prematurity (gestational age <26 weeks), timing of postnatal infection, as well as co-morbidities. Further research in postnatally infected preterm infants at risk for severe symptoms is essential with respect to preventive measures involving the infected breast milk and antiviral treatment.

Introduction

Infection with the double DNA-stranded cytomegalovirus (CMV) results in lifelong latency and subsequent chronic shedding through intermittent reactivation.[1] Neonates may acquire CMV in utero (congenital CMV infection, birth prevalence of 1.4%) after primary CMV infection of the mother or reactivation/reinfection in CMV seropositive mothers. Also, theoretically, CMV may be acquired through contact with cervico-vaginal secretions during passage through the birth canal (perinatal CMV infection).[2,3] Postnatal CMV infection is mostly acquired through CMV infected breast milk of seropositive mothers, with transmission rates up to 76% in all neonates.[4,5] In the preterm population, breast milk-acquired CMV infection has a median incidence of 20% (range 6-59%).[6]

Postnatal CMV infection is common in newborns. Although symptomatic postnatal CMV infections in term infants are rare, very preterm (<32 weeks gestational age) or very low birth weight (VLBW) infants are prone to develop symptomatic postnatal CMV infection. At present, there are no data to help predict which preterm infant will be at risk for a (severely) symptomatic postnatal CMV infection, potentially with long-term sequelae. As a consequence, it is not possible to selectively apply preventive measures involving CMV infected breast milk. Moreover, no global consensus exists on antiviral treatment of (severe) symptomatic postnatal CMV infection.

Thus, the focus of this review is to:

- Describe the epidemiology and long-term sequelae of postnatal CMV infection
- Summarize risk factors of postnatal CMV transmission in preterm infants
- Describe current predictors for severity of symptomatic postnatal CMV infection and propose suggestions for other potential predictors
- Describe the application of preventive measures and antiviral treatment in symptomatic postnatal CMV infection

Epidemiology and transmission

A postnatal CMV infection is the most common viral infection in neonates, with an estimated prevalence of 20 % (range 6-59%) among preterm infants.[6] Postnatal CMV infection can occur through contact with CMV-infected fluids such as breast milk, blood products, saliva or urine.[7–9] It has been widely recognized that breastfeeding strongly influences the epidemiology of postnatal CMV infections.[8,10] Transmission of CMV through CMV-infected blood transfusions has been virtually eliminated through the use of leuko-depleted CMV-negative blood products.[11]. Limited indirect data suggest that postnatal CMV infection may also be acquired through CMV infected secretions in the maternal birth canal.[3,12]

However, the role of intrapartum CMV transmission has been discussed based on negative ear- and throat swab testing directly after birth in postnatally infected infants and the absence of significant associations between vaginal delivery (versus caesarean section) and postnatal CMV infection.[8,13–16]

The transmission of CMV through breast milk requires a local reactivation of latent CMV in the mammary glands parallel to the start of lactation, through a mechanism that is largely unknown but may involve changes in cytokine concentrations.[10,17] The immune response to both primary CMV infection and CMV reactivation involves both innate immunity (including cytokine production and NK-cell induction) and adaptive immunity (including antibody production and CD4+ and CD8+ T cells induction).[18] It is of great interest that CMV specific CD8+ T cell populations have been determined at greater frequencies in breast milk as compared with blood,[19] which may underline the crucial role of CD8+ T cells in e.g. the cessation of virolactia (detection of infectious virus in the breast milk). In 96% of seropositive breastfeeding mothers, CMV DNA was eventually detected in breast milk.[8] Therefore, the reactivation rate almost equals the seroprevalence rate amongst breast feeding mothers. Upon reactivation and shedding of virus into the breast milk, breastfeeding mothers can be either transmitters in which breastfeeding will lead to a primary postnatal CMV infection in the infant, or non-transmitters in which exposure to CMV infected breast milk does not lead to infection in the infant.[10,20]

Diagnosis of postnatal CMV infection

A CMV polymerase chain reaction (PCR) of a urine, blood or saliva sample is currently the most commonly used method to determine a postnatal CMV infection. Before the introduction of the CMV-PCR, a viral culture method using monoclonal antibodies was used to determine CMV infection. A positive CMV culture or CMV-PCR of a sample taken within three weeks postpartum indicates a congenital CMV infection. Still, in postnatally infected preterm infants, a positive CMV-PCR in serum has been described at day 12 postpartum and viruria (positive CMV-PCR of urine) may start at 27 days postpartum.[8] At present, there is no gold standard for the diagnosis of postnatal CMV infection. However, a negative sample taken within three weeks postpartum and thereafter a positive sample is indicative of a postnatal CMV infection.[9] This diagnostic standard is used in most studies reported so far.

Clinical course of a postnatal CMV infection

There is no clear definition that encompasses the clinical course of a postnatal CMV infection in preterm infants. The risk of a symptomatic postnatal CMV infection in

preterm infants has been reported to be 0.7% (range 0-13.8%)[6] and thus, the majority of postnatally infected preterm infants are asymptomatic.[6,11] However, symptomatic infants may not be diagnosed or classified as symptomatic due to the transient nature of the symptoms (e.g. spontaneously resolving thrombocytopenia as the only symptom). A symptomatic postnatal CMV infection generally becomes evident between 35-60 days postpartum.[8,21] However, it is difficult to ascertain an average time of onset, as the moment of CMV reactivation and infectious shedding into breast milk and subsequent primary infection shows great inter-individual variability between mother-infant pairs and is often unknown.[22] Term born neonates have a very low risk of symptomatic postnatal CMV infection, possibly due to the transplacental transfer of protective anti-CMV antibodies, which increases with gestational age (GA), as well as the maturation of the foetal immune system.[23]

CMV is able to affect various organ systems with varying degrees of severity, leading to a morbidity that ranges from mild and self-limiting to overt and life-threatening.[8,9,14] Mortality in postnatal CMV infection in preterm infants is rarely reported in the literature.[24–26]

Clinical signs and symptoms

CMV sepsis-like syndrome or CMV sepsis-like symptoms (CMV-SLS) in preterm infants are commonly defined as the (aspecific) triad of apneas, bradycardia and gray pallor of the skin and is the most common clinical presentation of symptomatic postnatal CMV infection.[6,14] In a recent systematic review with population-based modelling by Lanzieri et al.[9], it was estimated that only 4.5% of VLBW or preterm infants with postnatal CMV infection develop CMV-SLS, which corresponds to approximately 2000 infants per year in the USA.

Other commonly described signs and symptoms of postnatal CMV infection with unknown prevalence, include acute hepatitis[14], hepatosplenomegaly and CMV pneumonia.[27] CMV enterocolitis[26], jaundice[6], cholestasis,[21,28] petechiae,[9] and lymphadenopathy[6] are infrequently associated with postnatal CMV infection. A recent analysis of clinical outcome in a cohort of preterm infants with postnatal CMV infection noted an increased incidence of spontaneous gastrointestinal perforations when compared to negative controls (13% versus 2%, respectively).[29]

Laboratory abnormalities

Thrombocytopenia is the most frequently reported laboratory abnormality in postnatal CMV infection.[6] Neutropenia, elevated liver enzymes and (mild) elevation of C-reactive protein are other reported abnormalities in blood parameters.[6,21,28] Frequently, blood abnormalities are the only manifestation of symptomatic postnatal CMV infection and only found coincidentally during standard clinical care of the preterm infant. Although many infectious agents may induce these abnormalities, they may be the reason to perform CMV diagnostics, especially when the infant also has clinical signs and symptoms.

Neuro-imaging

Serial analysis of cranial ultrasonography from birth until hospital discharge have shown that the development of lenticulostriate vasculopathy (LSV) at term-equivalent age [TEA] (i.e. 40 weeks postmenstrual age) was significantly associated with postnatal CMV infection in preterm infants.[30] In term infants with congenital CMV infection, LSV recognized with cranial ultrasonography was present in 54.3% of the investigated infants.[31] The exact causal mechanism behind this correlation requires further research, but it is has been suggested that CMV infection of the susceptible (preterm) brain may lead to necrotizing inflammation and subsequent mineralization of the wall of the lenticulostriate arteries.[32]

Recently, two articles were published in which diffusion tensor imaging and functional magnetic resonance imaging (fMRI), were used in preterm infants with postnatal CMV infection and compared with non-infected (pre-)term infants. At TEA, differences in the white matter microstructure were found in the occipital region.[33] In the other study by Dorn et al.[34], it was shown using fMRI that former very preterm and postnatally CMV infected infants employed different activational mechanisms when performing language and visuospatial tasks, compared to former non-infected preterm and healthy term-born infants.

Long-term sequelae

Sensorineural hearing loss

Sensorineural hearing loss (SNHL) has not been reported amongst infants with a postnatal CMV infection. Audiological assessment of infants with a postnatal CMV infection shortly after birth, at TEA, first year, second year and at the age of 4.5 years showed no indications of abnormal hearing,[27,35] although SNHL has been described in rare case reports.[36] In a recent study involving extremely preterm infants (22-24 weeks GA) with a postnatal CMV infection, 55% failed hearing examination at discharge.[37] However, upon further audiological examination, no infants had a hearing loss >20 dB (characteristic for SNHL). Permanent and late onset hearing loss do not seem to be associated with the clinical course of a postnatal CMV infection in preterm infants.

Neurodevelopmental outcome

Because of limited studies with small patient numbers, there is no clear consensus on the long-term neurodevelopmental outcome of preterm infants with a postnatal CMV infection. However, the majority of studies suggest that long term outcome of postnatal CMV infection in preterm infants is within the normal range, although infected infants may have impaired cognitive and motor scores when compared to non-infected infants.[6,38]

The most unfavorable long-term results are from a 1985 case-control study that followed term and preterm infants with postnatal CMV infection, up until the age of 3 years. A significantly more frequent development of neuromotor and cognitive impairment in infants with postnatal CMV infection[39] was reported. Three out of 13 postnatally infected premature infants had severe neuromotor impairment and four had severe handicap (DQ<70, neuromotor impairment or loss of vision or hearing). Of the control group none of the 13 infants had a severe neuromotor impairment, two had severe handicaps and another two had an impaired cognitive score. The association of these sequelae was correlated to early CMV excretion (<8 weeks postpartum) in urine and saliva and severe cardiopulmonary disease.[39] Premature infants considered "late excretors" (≥8 weeks postpartum) did not develop any sequelae. It is important to note that this study was carried out in the pre-surfactant era and that due to increased respiratory morbidity, more and worse symptomatic postnatal CMV infections may have been observed.

Subsequent studies that compared cognitive and motor outcome at various ages between postnatal CMV positive infants and their negative controls have found no profound short/long term implications.[16,27,34,35,38,40] However, a recent small case-control study investigating neurocognitive function at 8 years of age found significant differences between postnatal CMV positive and postnatal CMV negative children.[38] Mean scores on performance were within the normal range, but at the lower end of the spectrum. They also noted a higher percentage of infants in the postnatal CMV positive group that received a certain type of assistance at school. Interestingly, one of the most pronounced differences was noted in tests of visual processing, simultaneous integration and processing of information, as well as performance on fine motor dexterity.[38] The previously mentioned study of Dorn et al.[34], was the first study demonstrating long-term neurobiological consequences of an early postnatal CMV infection using functional MRI. However, the implication of these discrepancies and their impact on the overall quality of life are still unclear.

Infants with a postnatal CMV infection have already completed gross morphological development of the brain by the time of infection. Key processes of myelination, synaptic formation and neuronal growth are, however, far from complete. It is therefore, possible that short term analysis of neurodevelopmental outcome will not show any differences between the groups,[33,35] but that functional discrepancies may only become apparent at a later age when neurocognitive development can be assessed more reliably.[38,41] To draw more concrete conclusions about the long-term sequelae of postnatal CMV infection, larger prospective studies are needed.

Risk factors of postnatal CMV transmission

Several viral (e.g. CMV load and early shedding of CMV DNA and whole virus in breast milk), transmission route (e.g. breastfeeding) and host (e.g. GA) factors have been associated with a higher risk of postnatal CMV transmission from mother to child.

In the majority, postnatal CMV infection occurs in CMV seropositive mothers with the rare exception of infection through e.g. infected donor breast milk. In women who are seronegative for CMV, the risk of transmission of CMV through shedding in breast milk is nil.[8,13] Therefore, CMV seroprevalence among puerperal women, which is largely geographically and culturally determined[1], is the most important risk factor for postnatal CMV infection.

Viral and transmission route risk factors

In seropositive breast feeding mothers, early detection of viral DNA (DNAlactia) and infectious virus (virolactia) in breast milk whey, have been associated with a higher mother-to-infant transmission rate.[8,22] On average, transmitting mothers (mothers with CMV positive breast milk leading to infection in the infant) had DNAlactia and virolactia at a significantly earlier time after delivery when compared to non-transmitting mothers (mothers with CMV positive breast milk not leading to infection in the infant) (3.5 versus 8 days, p=0.025 and 10 versus 16 days postpartum, p=0.005, respectively).[8] Quantitative analysis of CMV DNA copy numbers taken from successive samples of infected breast milk over time show that transmitting mothers have a significantly higher CMV DNA load in their breast milk when compared to non-transmitting mothers.[20] In addition to higher viral load, viable CMV shedding persists for a longer time in transmitting mothers.[20]

Mother-to-infant transmission via breast milk does not solely require virolactia to be present in breast milk. Transmission and subsequent symptomatic postnatal CMV infection has also been reported to occur with only the detection (positive PCR, negative culture) of DNAlactia.[8] However, this may be explained by the lower sensitivity of the CMV culture.

There may also be an association between the start and duration of breast feeding.[15,42] Infants receiving fresh breast milk before 30 days of age and for a period longer than one month had a higher CMV infection rate than the control group (OR=4.5 [95% CI 1.14 - 17.6]; p=0.02).[15] However, it may also be possible that increased transmission is due to early CMV reactivation in breast milk and not due to the duration of CMV exposure.[14]

Infant host risk factors

When looking at serology, maternal and neonatal anti-CMV IgG levels are higher at birth amongst postnatal CMV infected infants when compared to non-infected infants.[40,43] A more recent study however, with a higher inclusion of infant-mother pairs found that absolute mother and infant anti-CMV IgG levels were not related to an increased risk of a postnatal CMV transmission.[44] Rather, low anti-CMV IgG infant-mother ratios (a measure of maternal-to-infant antibody transfer) were indicative of a higher risk of transmission. This finding was correlated to infants with a lower GA. The developmentally immature preterm immune system may facilitate viral pathogenicity, although the exact mechanisms remain unclear.[45,46] It has been proposed that subsets of natural killer cells are up-regulated in infants with postna-

tal CMV infection, reflecting an inadequate T-cell response.[46] Despite heavily relying on innate immunity, antiviral cytokines such as IFN-alpha are produced in limited amounts in preterm infants.[47]

Predictors of severity of symptomatic postnatal CMV infection

In the absence of a clear definition of a symptomatic postnatal CMV infection, it is difficult to differentiate between severely and mildly symptomatic postnatal CMV infections. However, physicians do make decisions regarding acute antiviral treatment based on the severity of the infection and/or the co-morbidity of the infant. In previous studies, severe symptomatic postnatal CMV infection involved clinical signs and symptoms of postnatal CMV infection and/or clinical deterioration of the infant. Mild symptomatic postnatal CMV infection included isolated laboratory abnormalities, such as thrombocytopenia. Factors that are predictive for more severe symptomatic postnatal CMV infection are increasingly recognized and include GA and time of viral detection. Urine and saliva CMV load and differences in CMV genotype seem less suitable as a severity predictor.

Gestational age and birth weight (BW)

The main established predictors of severe symptomatic postnatal CMV infection are (extremely) preterm birth and VLBW (<1500 grams).[8,9,14,20,37] Although these predictors are closely correlated, some studies only describe an association between one of both factors and severity of postnatal CMV infection.

GA seems to correlate strongly with a more detrimental clinical course. One of the first studies looking at the effects of GA found that infants who acquired postnatal CMV infection before 2 months postpartum had a much lower GA (GA 24.4 ± 0.5 weeks) in comparison to the group infected at 3 months postpartum or later (GA 30.1 ± 2.3 weeks).[13] Interestingly, 4/5 infants infected before 2 months postpartum had CMV-SLS, respiratory deterioration, leukopenia, thrombocytopenia and high concentrations of C-reactive protein. In contrast, 5/8 infants infected 3 months postpartum or later had mild laboratory abnormalities and 3/8 infants were asymptomatic.

A more recent study[37] retrospectively reviewed a group of postnatally infected extremely preterm infants (GA 22-24 weeks, mean BW 541 grams). Infants in this study received untreated fresh breast milk and the incentive to do CMV diagnostics was the initial occurrence of thrombocytopenia. In these infants there was a high incidence of severe symptomatic postnatal CMV infection (65%) including CMV-SLS (55%), which is much higher compared to symptomatic infection in preterm infants with GA 24-32 weeks (median 0.7%, range 0-13.8%).[6] No mortality or SNHL was reported. The manifestations of the symptoms were strongly related to GA, BW and co-morbidities present at birth.

Another study described a significant correlation between BW and symptomatic manifestation, indicating that the lower the BW, the earlier the first day of viral detection in the infant and subsequently the higher the chance of a symptomatic postnatal CMV infection (48% [16/33] of the infants with postnatal CMV infection showed at least 1 symptom).[14] They calculated a 3.58 fold increase (OR 3.58 [95% CI 1.00-12.68]) in risk of primary symptomatic transmission in VLBW infants. A higher BW correlated with later viral detection and a mild or asymptomatic clinical course.[14]

Potentially supporting these findings is a case series study,[24] in which over a two-year period five infants with a median GA of 27 weeks and a median BW of 860 grams receiving fresh untreated breast milk within 1-2 weeks postpartum presented with fulminant disease and mortality. More recently, it has been suggested that in VLBW infants with an asymptomatic postnatal CMV infection that are clinically stable and growing well, an uneventful clinical course can be expected.[48] However, with underlying pathology (chronic lung disease) prior to infection, severe symptomatic manifestation may occur (CMV-SLS, increased oxygen requirement, abdominal distension). In all, extremely preterm infants (e.g. GA < 26 weeks) with a postnatal CMV infection appear to have the highest risk of severe symptomatic postnatal CMV infection.

Time of viral detection

Another predictor of symptomatic postnatal CMV infection severity may be the postnatal age of the infant at the time of first virus detection in urine or blood.[8,14] In an instrumental logistic regression analysis, Maschmann et al.[14] demonstrated that the age of the infant at the time of first virus detection in urine correlated with the risk of symptomatic infection and depended on BW. They observed a 1.39-fold increased risk of a symptomatic virus transmission with every reduction in BW by 100 grams and a 2-fold reduction in the time until the virus is first detected, increased the risk of symptomatic evolution by 3.58-fold.[14] The authors suggest that preterm infants with a greater BW and delayed transmission event are more able to cope with a primary postnatal CMV infection and therefore, will experience an asymptomatic course. In line with this suggestion, although not clearly specified in the article, time of first virus detection may also be predictive of severity of symptomatic postnatal CMV infection. This association was also suggested in another article, where it was shown that infants who have onset of viruria before a GA of 32 weeks have a higher risk of developing CMV-SLS.[9]

Pre-existing morbidity

The physiology of the preterm infant naturally predisposes it to a high pre-infection morbidity mainly due to an underdeveloped immune system and potential inflictions of vital organ systems that are not functioning optimally. It is not possible to ascribe this functional difference in immunity to individual components. It is a combination of a lack of protective maternal antibodies, numerical difference in circulating white blood cells and relatively reduced amounts of anti-viral responses.[47] In this context, it has been proposed that CMV is more of a co-actor that aggravates the clinical

course associated with prematurity.[28,37] In other words, in selected preterm infants with severe co-morbidity, postnatal CMV infection may aggravate the clinical course of a pre-existing condition, but it is unlikely to cause symptoms in otherwise healthy preterm infants. Although co-morbidities or pre-existing conditions are a likely severity predictor of symptomatic postnatal CMV infection, it is largely confounded by GA, as extreme prematurity (i.e. < 26 weeks) is generally associated with (severe) co-morbidity.

Viral load

Jim et al.[20] found in their cohort of VLBW infants with breast milk acquired CMV that 3/5 (60%) infants with a symptomatic postnatal CMV infection had higher CMV loads in their urine compared to asymptomatic infants. However, this study was limited by its population size. In a study investigating the efficacy of screening saliva compared to urine in preterm infants with postnatal CMV infection,[49] the mean viral load in saliva and urine between 9 symptomatic infants and 38 asymptomatic infants was comparable and not statistically different (Figure 1, unpublished data). These data are in line with previous findings, where viral load was not associated with the presence of CMV-associated cerebral abnormalities.[50] In breast milk, a relation between CMV load and presence of symptomatic postnatal CMV infection has not been established.[21]

In the absence of an association of viral load with symptomatic postnatal CMV infection, it seems unlikely that viral load in urine, saliva or breast milk can be used as a predictor for severity.

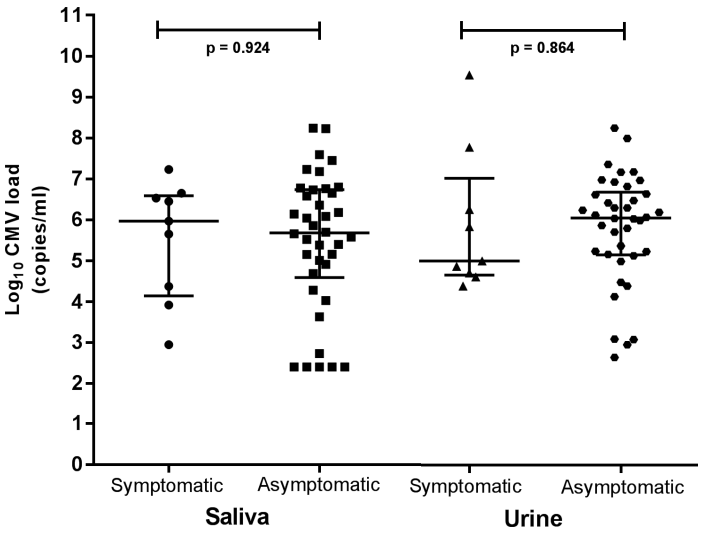

Figure 1. Log_{10} CMV load in saliva and urine categorized in symptomatic and asymptomatic postnatal CMV infection in preterm infants. Bars represent median viral loads and whiskers represent 25th and 75th percentile (unpublished data, figure derived from Gunkel et al.[49]).

CMV genotypes

In congenital CMV infection, it has been reported that genetic diversity may influence the clinical manifestation of CMV disease. Genetic variation in the CMV genome has commonly been reported in the UL144 tumor necrosis factor alpha-like receptor gene, UL55 envelope glycoprotein B (gB) gene, UL73 envelope glycoprotein N (gN) gene, UL75 envelope glycoprotein H (gH) and US28 beta-chemokine receptor gene.[51–56] Two studies have (also) reported genetic diversity in postnatally infected infants. Yan et al.[54] found no correlation between UL55 (gB) diversity and clinical manifestation of the disease. In a recent study by Paradowska et al.[56] it has been suggested that UL73 (gN) type 2 and 4 genotypes may induce a more severe clinical manifestation. However, the results of both studies are limited by the fact that congenital CMV infection has not been excluded in all postnatally infected infants and reported symptoms (including hearing loss, CNS damage and psychomotor retardation) have not previously been associated with postnatal CMV infection.

Therefore, no definite conclusions regarding CMV genotypes and severity of symptomatic postnatal CMV infection can be drawn. However, in congenital CMV infection, genetic diversity seems promising as a severity predictor. In postnatally infected infants, more research which may include whole genome sequencing has to be performed.

Suggestions towards the identification of other predictors

Globally, the GA on which extremely preterm infants are actively treated is decreasing with a current minimum of 22 weeks in e.g. the USA, Scandinavia and Germany. This may result in more severe symptomatic postnatal CMV infections and may eventually worsen the global neurodevelopmental outcome of this already vulnerable patient group.[37] The extremely preterm infant (<26 weeks GA) population is relatively new and not well characterized yet. It would be very interesting to commence a prospective cohort study on clinical characteristics and neurodevelopmental outcome of these infants, including screening for postnatal CMV infection. Studying a sufficiently large cohort of preterm infants with symptomatic postnatal CMV infection may clarify the hypothesis whether postnatal CMV infection aggravates a pre-existing condition or the pre-existing condition facilitates a more severe symptomatic postnatal CMV infection.

More research is needed to understand the pathophysiological mechanisms that gauge severity of postnatal CMV infection. It is still unclear whether viral characteristics (e.g. specific genotypes, viral load, differences in protein expression) and/or host factors (e.g. stage of development of the immune system, genetic differences (toll-like receptors, T-cells)) are responsible for the development of symptomatic infection. It has been increasingly recognized that innate immunity responses are key in early postnatal infections.[57] Genetic differences in innate immunity may also influence the course of symptomatic postnatal CMV infection. Recently, it has been suggested

that epigenetic differences in toll-like receptor 2 (TLR-2) and TLR-4 polymorphisms may be associated with congenital CMV infection and its clinical manifestation.[58,59] In congenital CMV vaccine development, it has been shown that TLR polymorphisms influence anti-CMV antibody production,[60] but may differ age-dependently. Because methods to identify epigenetic differences and genetic polymorphisms are rapidly evolving, research regarding the relation between (severity of) CMV infection and innate immunity can be expected.

Preventive measures and antiviral treatment

Prevention of CMV transmission

Severe symptomatic postnatal CMV infection may be prevented by treating or withholding infected breast milk. In the general population of preterm infants, the American Academy of Pediatrics suggests that the value of using fresh breast milk from seropositive mothers outweighs the risk of postnatal CMV infection.[61] However, in Germany, Austria and Switzerland untreated fresh breast milk is preferably not given to preterm infants <32 GA or <1500gr BW because of the risk of postnatal CMV infection.[62] Various measures can be implemented to reduce the risk of transmission via breast milk. As suggested by Hamprecht et al.[10], an essential first step in prevention of CMV transmission is to determine maternal anti-CMV serostatus to identify the infant at risk of breast milk-acquired infection. In seropositive mothers, CMV (DNA) load in the breast milk may be monitored postpartum quantitatively by using real-time polymerase chain reaction (rtPCR) once a week. When virolactia and viral DNAlactia in breast milk are at their maximum (mostly at 4–5 weeks postpartum) CMV transmission is most likely.[10,13,21,63] However, in practice, monitoring CMV load in breast milk by rtPCR) on a weekly basis in all infants at potential risk seems like a costly and time-consuming method.

To reduce or completely deactivate the infectious capacity of the virus, the infected breast milk can be freeze-thawed or heated (pasteurization). Freezing of the breast milk and/or subsequent cycles of freeze-thawing reduces CMV load in the breast milk but does not sufficiently protect against CMV transmission.[42,64] However, transmission rates were lower (6.2% using predominantly frozen milk at -20º Celsius[42] versus 37% using only fresh breast milk[64]) and the beneficial properties of breast milk were preserved. Pasteurization successfully inactivates CMV in the breast milk, but also influences the composition of breast milk, which depends on the manner of pasteurization.[65–67] Holder pasteurization (heating at 63° Celsius for at least 30 minutes) combined with freezing at -20° Celsius is widely used to eliminate CMV. However, this method also results in a significant decrease in energy, fat and lactose content of the breast milk.[67] High temperature – short time pasteurization (heating at 72° Celsius for 16 seconds) is another method.[65] This method compared to Holder pasteurization indicates better preservation of beneficial components, such as AP and lipase activity[64] as well as growth factors.[66]

Treatment of postnatal CMV infection

To date, there are no controlled studies that have evaluated the efficacy and safety of antiviral medication or anti-CMV immunoglobulins in the treatment of a symptomatic postnatal CMV infection in preterm infants. As a result, there are no generally accepted treatment recommendations for this patient group so far.[68]

Antiviral treatment, i.e. ganciclovir (Cymevene®), is known to cause hematological and carcinogenic toxicity as well as infertility in animal studies.[69] Studies have reported the occurrence of cholestatic side effects,[10] persistent neutropenia[69,70] and bone marrow suppression.[69] The efficacy of ganciclovir amongst neonates has been studied in a randomized controlled trial in term infants with congenital CMV infection.[69] A lower incidence of SNHL was shown in the intervention group. However, the antiviral treatment was associated with severe neutropenia (63% in the intervention group versus 21% in the control group, $p<0.01$) and the results may have been biased by the extensive loss to follow-up.

Another study documented better neurodevelopmental outcome in ganciclovir-treated infants with congenital CMV infection and involvement of the central nervous system, compared to infants who received no antiviral therapy.[71] However, the results of these studies are limited to term infants with congenital CMV infection with central nervous system involvement and cannot be extrapolated to preterm infants with symptomatic postnatal CMV infection.

In preterm infants with severe (i.e. life-threatening) symptomatic postnatal CMV infection, the incidental use of ganciclovir and valganciclovir (Valcyte®) has been reported.[24,37,70] Clinical course of infection and co-morbidities of the infant were considered before administration of antiviral medication. Antiviral treatment was given in the form of intravenous ganciclovir or oral valganciclovir for 4-6 weeks on average. Subsequently, it was observed that treatment allows clinical and especially hematological parameters to normalize again.[10,37] After cessation of therapy, asymptomatic viral shedding may be observed.[10,70] In the recent study by Mehler et al.[37] long-term outcome of the reported treated cases was comparable to non-infected extremely preterm infants and no SNHL occurred.

There are sporadic reports on intravenous administration of intravenous immune globulin (IVIG), containing anti-CMV antibodies, to postnatally infected (and symptomatic) preterm infants.[21,72,73] Titers of anti-CMV antibodies in IVIG are high, but this may differ depending on the region of donation. Only one placebo-controlled trial using IVIG was performed (at the time preterm infants did not yet receive CMV-negative blood transfusions),[72] which documented a lower incidence of CMV-SLS (3.2% versus 12.5% in the placebo group, respectively). It has been hypothesized that administration of IVIG in a selected population and timeframe (2–6 weeks postpartum) might eventually prevent or in a later timeframe, treat symptomatic postnatal CMV infection.

CMV employs different glycoprotein complexes to enter target cells, using gB or gH-gL-gO complexes for entry into fibroblasts, whereas the gH/gL/UL128/UL130/UL131 complex is primarily used for entry into endothelial cells and epithelial cells.[74,75] Current research suggests that the neutralizing capacity mediated by anti-CMV hyperimmune globulin primarily targets the gH/gL/UL128/UL130/UL131 glycoprotein complex of CMV, rather than the gB complex.[75] Advances in understanding the targets will aid in the development of suitable and target specific anti-CMV immune globulin therapy.

Application of preventive and therapeutic measures

More research is warranted to produce a globally accepted protocol for the prevention and/or treatment of symptomatic postnatal CMV infection in preterm infants. In view of the risk factors for CMV transmission, severity predictors of symptomatic postnatal CMV infection and risks of long-term sequelae, the most important factor seems to be extreme prematurity (<26 weeks GA). Therefore, in these infants, it is advisable to determine maternal serostatus. If the mother is CMV seropositive, pasteurization of the breast milk during the first 6-8 weeks of life using the high temperature – short time pasteurization[64] should be considered, especially in infants with severe co-morbidities, to ensure sufficient reduction in viral shedding and subsequently, transmission risk.

Severe, life-threatening symptomatic postnatal CMV infection in the extremely preterm infant (GA <26 weeks) may be treated using antiviral medication (i.e. ganciclovir and/or valganciclovir), in the absence of other treatment options.[21] However, the optimal dose, duration and the expected benefits of treatment are unknown.[68] If antiviral treatment is commenced, toxicity of the antiviral medication (e.g. neutropenia) should be closely monitored. Currently, no evidence in favor of the addition of IVIG to the antiviral medication has been published and the safety of this treatment has not been established. In general, it is recommended to individually tailor treatment specifications and carefully consider the benefits of starting treatment against the treatment-associated side effects.[69] Recently, Tengsupakul et al.[76] reported on case of postnatal CMV infection presenting with necrotizing enterocolitis in which they retrospectively analyzed serial dried blood spot cards (daily obtained for a metabolic study). Interestingly, asymptomatic DNAemia was observed prior to onset of symptoms. Although, not a conceivable method as yet, this novel approach may be realized in the future with the advent of point of care PCR assays, to identify high-risk infants who may benefit from pre-emptive treatment.

Expert commentary and five-year view

Postnatal CMV infection in preterm infants through infected breast milk is a global and common problem. Although the majority of the postnatal CMV infections remain asymptomatic or are associated with mild symptoms or laboratory abnormalities such as thrombocytopenia, some infants may develop severe and life-threatening disease. Recently, it was (again) acknowledged that extremely preterm infants (GA <26 weeks) are at high risk of symptomatic postnatal CMV infection. With infants born at lower GA (<24 weeks) symptomatic postnatal CMV infection may become an even more widespread problem.

More research is warranted to further characterize the infant at risk of severe symptomatic postnatal CMV infection and to work towards a clear and internationally applicable definition of symptomatic postnatal CMV infection.

With the continued effort in defining symptoms and identifying risk factors, predictors of severity and long-term sequelae, we will get a clear a picture of the infants needing treatment. Preventive and therapeutic measures may then be applied in specific patients and/or situations which should be guided by the clinical course of the disease. Based on current literature, we advise to determine maternal serostatus in the extreme preterm infant population (<26 weeks of gestation). Breast milk of CMV positive mothers of infants at risk of (severe) symptomatic CMV infection should then be pasteurized during the first 6-8 weeks of life, to reduce the risk of transmission, especially in infants with severe co-morbidities.

In the future, methods to pre-treat breast milk will have to be optimized and commercialized to reduce transmission, especially during the period of high viral load, to still allow the nutritional and immunological benefits of the breast milk.

Currently, a decision to treat symptomatic postnatally infected preterm infants with antiviral medication is unfortunately based on very limited evidence and is conceivably often fueled by discomfort amongst clinicians of abstaining from additional treatment. Therefore, a multi-centre trial should establish the efficacy, safety and optimal duration of antiviral medication in symptomatic postnatally infected preterm infants, with special attention to the long-term neurodevelopmental outcome.

Key issues

- Postnatal CMV infection acquired through infected breast milk, is common in preterm infants and is mostly asymptomatic.
- Several viral (e.g. CMV load and early shedding of CMV DNA in breast milk), transmission route (e.g. breastfeeding) and host (e.g. GA) factors have been associated with a higher risk of postnatal CMV transmission from mother to child.
- Signs and symptoms of symptomatic postnatal CMV infection include CMV sepsis like syndrome/-symptoms (apneas, bradycardia and gray pallor of the skin), hepatitis, CMV pneumonia, thrombocytopenia, neutropenia and CRP elevation.
- Long-term neurodevelopmental outcome of postnatal CMV infection in preterm infants seems favorable, although large prospective studies on this subject still have to be performed.
- Extreme prematurity (GA <26 weeks), time of primary infection and probably severe co-morbidity seem to be predictors of severity for symptomatic postnatal CMV infection in preterm infants.
- Identification of the preterm infant at risk for severe symptomatic postnatal CMV infection is essential for the application of preventive and therapeutic measures.
- Preventive measures may include freeze-thawing or pasteurization of infected breast milk or, eventually, withholding infected breast milk.
- Severe, life-threatening symptomatic postnatal CMV infection in the extremely preterm infant (GA <26 weeks) may be treated using antiviral medication (i.e. ganciclovir and/or valganciclovir), in the absence of other treatment options. However, the efficacy and safety of the antiviral medication has not been established in this population.

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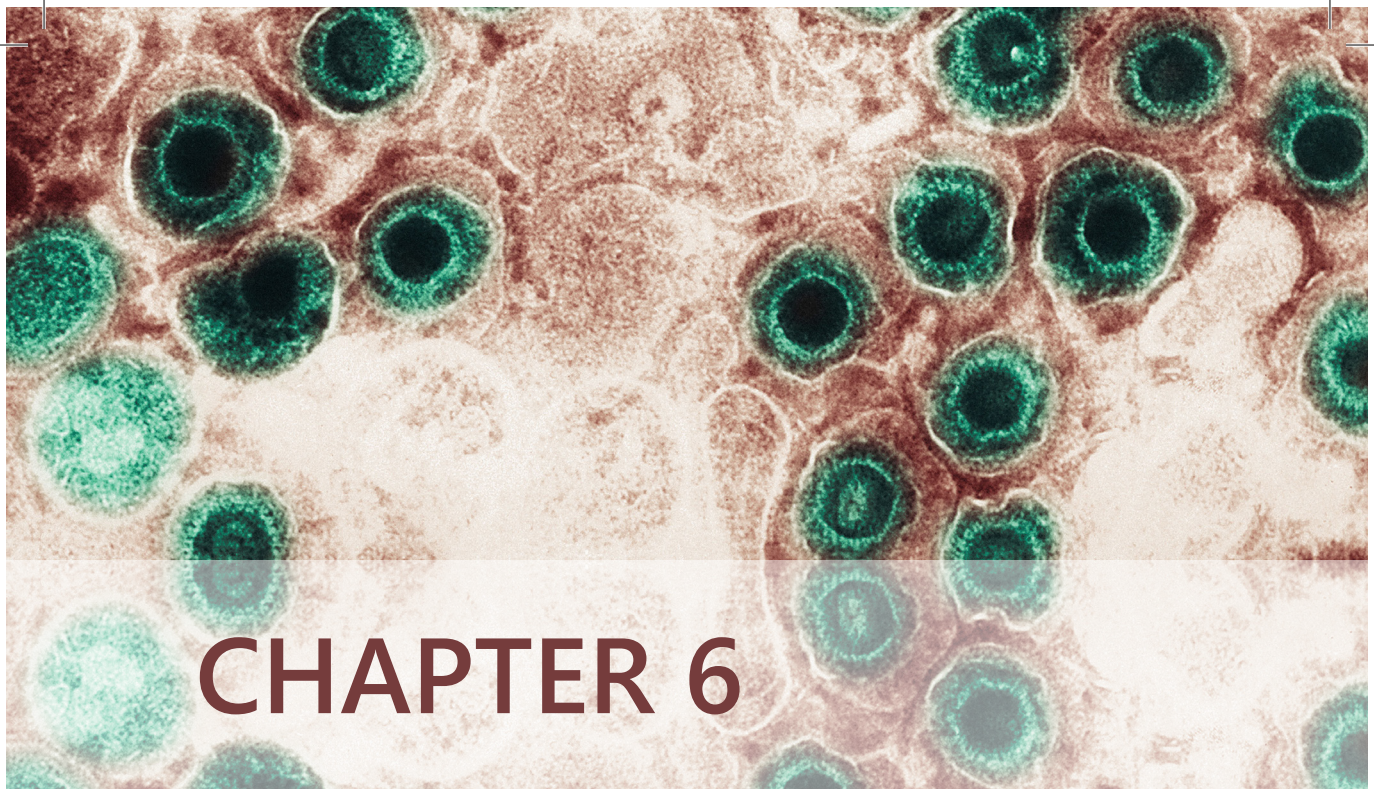

CHAPTER 6

Urine is superior to saliva when screening for postnatal CMV infections in preterm infants

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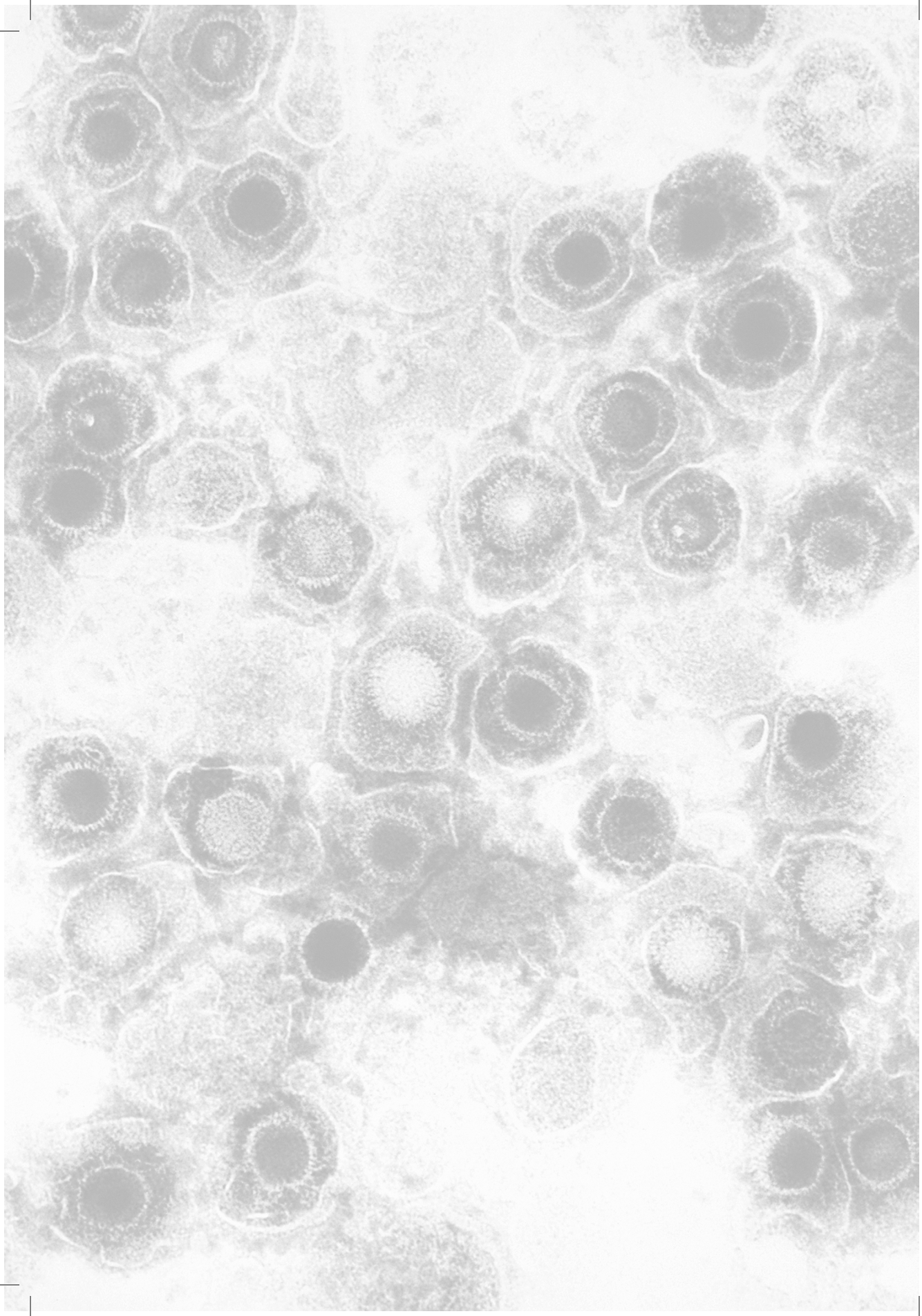

CHAPTER 6

Urine is superior to saliva when screening for postnatal CMV infections in preterm infants

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Abstract

Background Cytomegalovirus (CMV) is the most frequently contracted virus in preterm infants. Postnatal infection is mostly asymptomatic but is sometimes associated with severe disease. To diagnose an infection, urine or saliva samples can be tested for CMV-DNA by real-time polymerase chain reaction (rtPCR). Although the diagnostic accuracy of testing saliva samples has not been determined in preterm infants, saliva is widely used because it is easier to obtain than urine.

Objectives To determine whether screening of saliva is equivalent to urine to detect a postnatal CMV infection in preterm infants.

Study design Between 2010 and 2013 saliva and urine samples were collected from infants admitted to the Neonatal Intensive Care Unit of the University Medical Center Utrecht and born with a gestational age (GA) below 32 weeks. Urine samples were obtained within three weeks after birth and urine and saliva samples at term equivalent age (40 weeks GA) and tested for CMV-DNA by rtPCR. Infants with a congenital CMV infection were excluded.

Results Of 261 preterm infants included in the study, CMV-DNA was detected in urine of 47 and in saliva of 43 children. Of 47 infants with postnatal CMV infection, CMV was detected in 42 saliva samples (sensitivity 89.4%; CI 76.9 – 96.5). Of 214 children without postnatal CMV infection, one saliva sample tested positive for CMV (specificity 99.5%; CI 97.4-99.9).

Conclusions Screening saliva for CMV-DNA by rtPCR is inferior to urine to diagnose postnatal CMV infections in preterm infants.

Introduction

Human cytomegalovirus (CMV) is the most commonly contracted virus in preterm infants (born before 32 weeks gestational age) and is mainly acquired through CMV positive breast milk.[1] In one study of CMV acquisition among preterm neonates, the mother-to-infant transmission rate among CMV seropositive mothers was 37%.[1] Postnatal CMV infections (pCMV) are mostly asymptomatic, however disease severity appears to be inversely related to gestational age (GA) and birth weight (BW).[2–4] pCMV induced long-term sequelae seem less common compared to congenital CMV (cCMV), but neurocognitive impairment,[5] changes in white matter microstructure[6] and discrepancies in language and visuospatial functions detected by functional magnetic resonance imaging[7] have been reported. More studies are needed to determine the burden of disease of pCMV infections. Screening would allow for more clarity on short- and long-term neurodevelopment. CMV infections are diagnosed by viral culture of urine samples or detection of CMV-DNA by real-time polymerase chain reaction (rtPCR).[8–10] Studies show that saliva is suitable to screen infants for cCMV infections, which has the advantage of being easier to obtain than urine.[8,10,11] No studies have evaluated the sensitivity and specificity of saliva to screen for pCMV infections.

Objectives

This study was designed to assess the diagnostic value of screening saliva for CMV-DNA in preterm infants for a pCMV infection. Results were compared with screening of urine, which was regarded as the gold standard for cCMV infection.[12]

Study design

Patient inclusion and specimen collection

From March 2010 until June 2013 all preterm infants with a GA <32 weeks admitted to the Neonatal Intensive Care Unit of the University Medical Center Utrecht, The Netherlands were screened for a pCMV infection. Saliva and urine samples were collected at term-equivalent age (TEA = GA 40 weeks). Saliva was obtained by placing a Copan Flocked Swab (FLOQSwab, Copan Flock Technologies SrL, Brescia, Italy) inside the infant's cheek. Swabs were transferred to 3ml Universal Transport Medium (UTM, Copan, Italy) for processing. Urine was collected on sterile gauzes and placed in sterile beakers (Greiner Bio-one GmbH, Kremsmünster, Austria) without additives or in sterile urine bags (Urinocol Premature, B. Braun Medical, Boulogne-Cedex, France). Infants that died before TEA or with cCMV infection (CMV-DNA positive urine collected ≤3 weeks postpartum) were excluded. When urine was not available, dried blood spot (DBS) filter cards collected ≤5 days postpartum were tested. GA, gender, BW and feeding type (untreated breast milk or formula) were recorded. In-

fants with sepsis-like illness, thrombocytopenia and/or respiratory disease, negative viral diagnostics for common respiratory viruses, negative blood culture and positive CMV rtPCR of urine or plasma at time of symptoms were considered symptomatic.[13]

Quantitative Real-Time PCR

Urine and saliva were tested for CMV-DNA by quantitative rtPCR, which is an adapted, internally controlled rtPCR as previously described,[14] with a 250 copies/ml quantification limit. Briefly, viral DNA was extracted from 200µl urine or 200µl saliva in UTM using the MagnaPureLC system (MPLC, Roche Diagnostics, Penzberg, Germany) and Total Nucleic Acid isolation kit for MPLC (Roche Diagnostics, Penzberg, Germany). For urine collected after July 1, 2012, total nucleic acid extraction was performed using MagnaPure-96 system (Roche). Upon elution of the DNA in 100µl elution buffer, 10µl of DNA eluate was tested for CMV-DNA by rtPCR.[14] Cycle thresholds from positive rtPCR reactions were converted into viral loads using an external standard curve. For DBS filters, nucleic acid was extracted from 3 circular punches of 7 mm^2 each, equal to approximately 20µl of blood, using the QiaAmpDNAmini kit (Qiagen, Hilden, Germany). Upon elution in 150µl of elution buffer, 10µl of the eluate was tested for CMV-DNA by rtPCR as described above.

Statistical analysis

Sensitivity and specificity was calculated with urine rtPCR as a reference. This was done for all infants and for asymptomatic and symptomatic infants separately.

Results

302 infants were enrolled during the study period. Forty-one/302 (13.6%) were excluded due to lack of paired saliva–urine samples (n=38), cCMV infection (n=2) or death before TEA (n=1). Mean GA and BW were 27.7 weeks [range 24.1-31.9] and 1076 grams [range 455-2130] respectively. Two-hundred-forty-five/261 (93.9%) infants received breast milk and 16/261 (6.1%) received exclusively formula. A pCMV infection was detected at TEA in 47/261 (18%) infants by rtPCR in urine and in 42/261 (16.1%) infants by rtPCR in saliva (Table 1). Plotting log10 urine CMV load against log10 saliva CMV load showed a weak positive correlation, p=0.0001 (Figure 1).

rtPCR in saliva failed to identify a pCMV infection in 5 infants (5/47= 10.6%). They were all asymptomatic, male, had a mean GA of 28.7 weeks (SD 1.8) and all received breast milk for ≥4 weeks before TEA. The median viral load in their urine (4.12 $\log_{10}$ copies/ml) was significantly lower compared to urine of infants with a positive result on both assays (6.01 $\log_{10}$ copies/ml) (n=42), p=0.033 (Figure 2). One infant had a positive saliva and a negative urine result. Nine/47 infants (19.1%) were symptomatic during admission. Symptoms included thrombocytopenia, leukopenia, increased liver transaminases and respiratory disease. During admission urine was tested on clinical indication at an average of 59.3 days (SD 16.0) postpartum, with a median

Table 1. Comparison of saliva rtPCR with urine rtPCR samples taken at TEA as a total cohort (n=261), only including symptomatic infants (n=223) and only including asymptomatic infants (n=252), in the detection of a postnatal CMV infection.

	Saliva rtPCR assay	**Urine rtPCR assay**	
	Positive	Negative	Total
Positive	42	1	43
Negative	5	213	218
Total	47	214	261
Reference: urine rtPCR			
Sensitivity (95% CI) – %	89.4	(76.9 – 96.5)	
Specificity (95% CI) – %	99.5	(97.4 – 99.9)	
Positive predictive value (95% CI) – %	97.7	(87.7 – 99.9)	
Negative predictive value (95% CI) – %	97.7	(94.7 – 99.3)	
Including only symptomatic infants			
Sensitivity (95% CI) – %	100	(66.4 – 100)	
Specificity (95% CI) – %	99.5	(97.4 – 99.9)	
Positive predictive value (95% CI) – %	90.0	(55.5 – 99.8)	
Negative predictive value (95% CI) – %	100	(98.3 – 100)	
Including only asymptomatic infants			
Sensitivity (95% CI) – %	86.8	(71.9 – 95.6)	
Specificity (95% CI) – %	99.5	(97.4 – 99.9)	
Positive predictive value (95% CI) – %	97.1	(84.7 – 99.9)	
Negative predictive value (95% CI) – %	97.7	(94.7 – 99.3)	

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urine viral load of 4.3 $\log_{10}$ copies/ml (compared to a median urine viral load of 4.99 $\log_{10}$ copies/ml at TEA p=0.17). In this group, rtPCR detected CMV-DNA in all saliva samples (sensitivity 100%). In asymptomatic pCMV positive infants, saliva rtPCR failed to detect CMV-DNA in 5/38 infants (13.2%).

Discussion

To the best of our knowledge this is the first study investigating sensitivity and specificity of screening saliva for CMV-DNA by rtPCR to diagnose postnatal CMV infections in preterm infants. Saliva has been an attractive candidate for replacement of urine due to its ease of acquisition, and has been used to screen for cCMV infections.[8,10,15,16] The results of our study show that screening of saliva for pCMV infections in preterm infants at TEA has lower sensitivity than urine. More than 10% of pCMV infections were missed, indicating that the results of saliva rtPCR should be interpreted with caution.

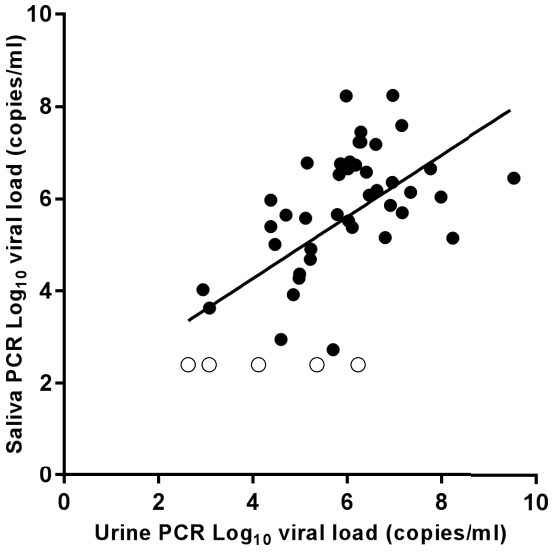

Figure 1. Scatterplot of log_{10} CMV loads (copies/ml) in urine and saliva at TEA from the pCMV positive infants (n=47). White circles indicate infants with a positive urine rtPCR and a negative saliva rtPCR (n=5). Pearson correlation coefficient: 0.58, p = 0.0001. Pearson correlation coefficient removing infants with a negative saliva rtPCR (n=42): 0.50, p = 0.0006.

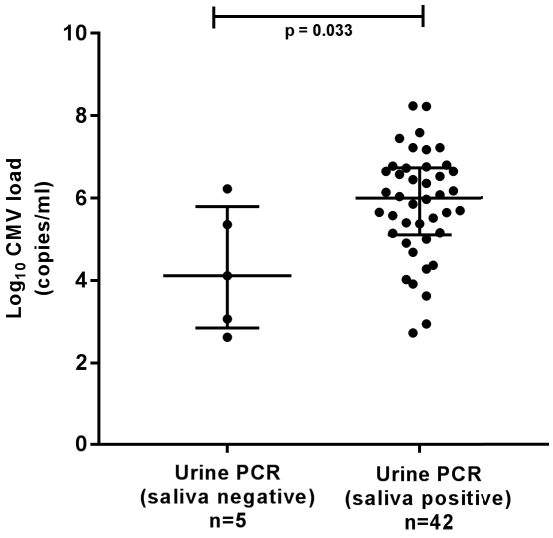

Figure 2. Urine log_{10} CMV load (copies/ml) in infants with a negative saliva rtPCR and infants with a positive saliva rtPCR. Central bars in scatter dot plot represent median viral loads after log_{10} transformation; whiskers represent the interquartile range.

Lower sensitivity may be attributed to lower mean viral shedding in infants with pCMV compared to infants with cCMV infection.[13,17] The median urine viral loads in infants with negative saliva and positive urine were significantly lower when compared to infants that had positive results on both assays. Unfortunately, follow-up saliva samples beyond TEA were not available. We cannot exclude that saliva would have become CMV-DNA positive at later time-points. A discrepancy between urine and saliva was seen in a study by K Hamprecht et al., 2001, where virus was detected earlier in urine than in throat swabs or tracheal secretions amongst symptomatic pCMV infected preterm infants. The result of the infant with a positive saliva and negative urine sample was probably due to contamination of saliva with CMV infected breast milk[18] cCMV infection was excluded on urine collected ≤3 weeks postpartum. The discrepant results may also indicate a difference in distribution of CMV in the body.[9,17,19] Studies on the kinetics of CMV excretion in saliva, in the context of CMV reactivation in adults and older children show more variation in duration and excretion of CMV in saliva when compared to urine.[20,21]

Interestingly, our sub-analysis showed a sensitivity of 100% for saliva rtPCR when including only symptomatic infants. Furthermore, swabs for saliva collection contained UTM and dilution could have resulted in lower sensitivity. Because saliva swabs were inserted into 3 mL of UTM before testing, saliva was diluted about 25 times. With rtPCR this dilution factor theoretically results in an increase of the CT-value with 4-5 points compared to undiluted material. Since all infants with a pCMV infection and a positive saliva sample had a CT-value below 36 (in other words, had strong positive test results) its seems unlikely that dilution would have led to a false negative test result. While a pCMV infection was determined using two measurements, a limitation is the uncertainty about the exact time of primary infection. Due to the predominately asymptomatic nature of the infection, a fixed time point was chosen for saliva sampling during a routine follow-up visit at TEA, ensuring enough time for primary infection to occur. Based on our findings we recommend using urine instead of saliva when screening for asymptomatic pCMV infections. For diagnostic purposes, however, saliva may be used to screen infants with clinical symptoms that may be due to pCMV infection.

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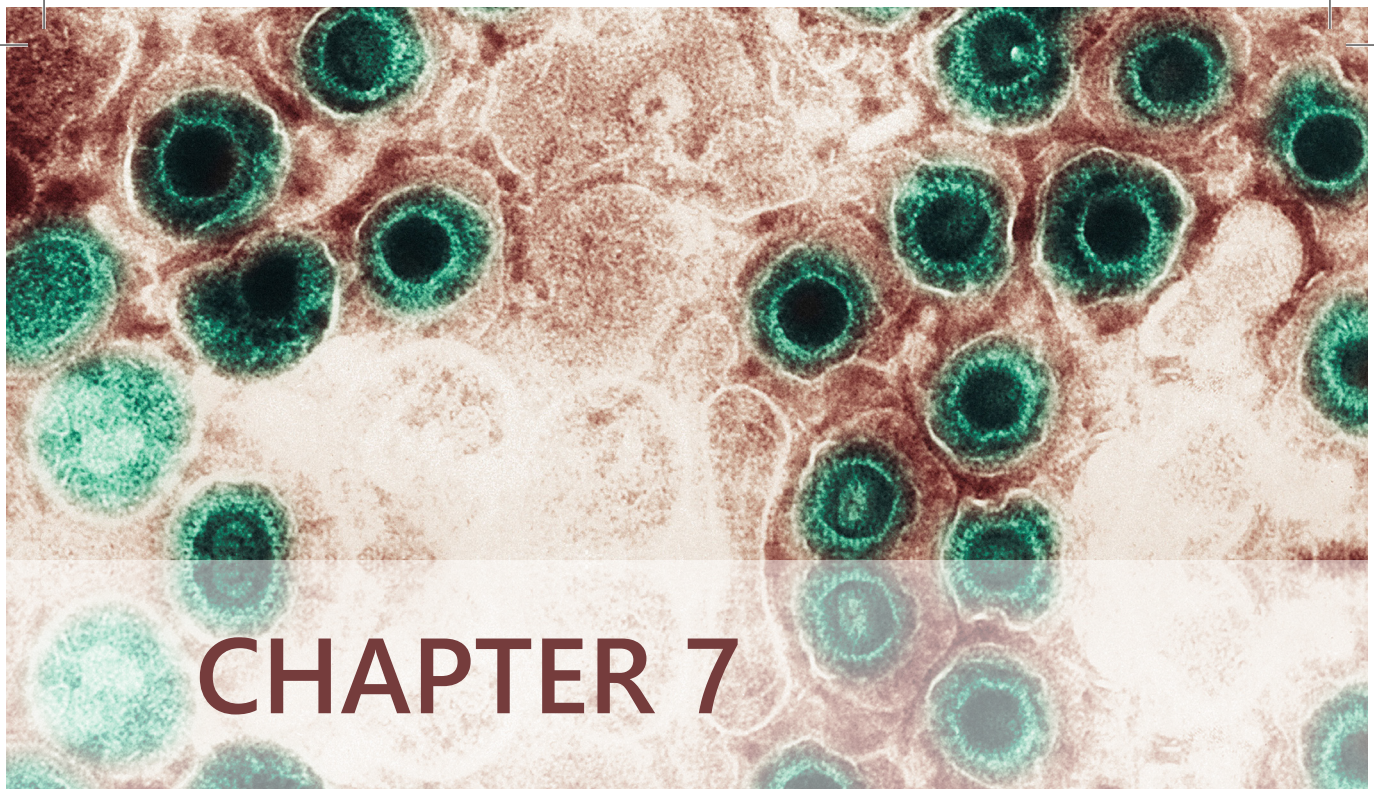

CHAPTER 7

Reduced occipital FA on cerebral diffusion tensor imaging in preterm infants with post-natally acquired cytomegalovirus infection

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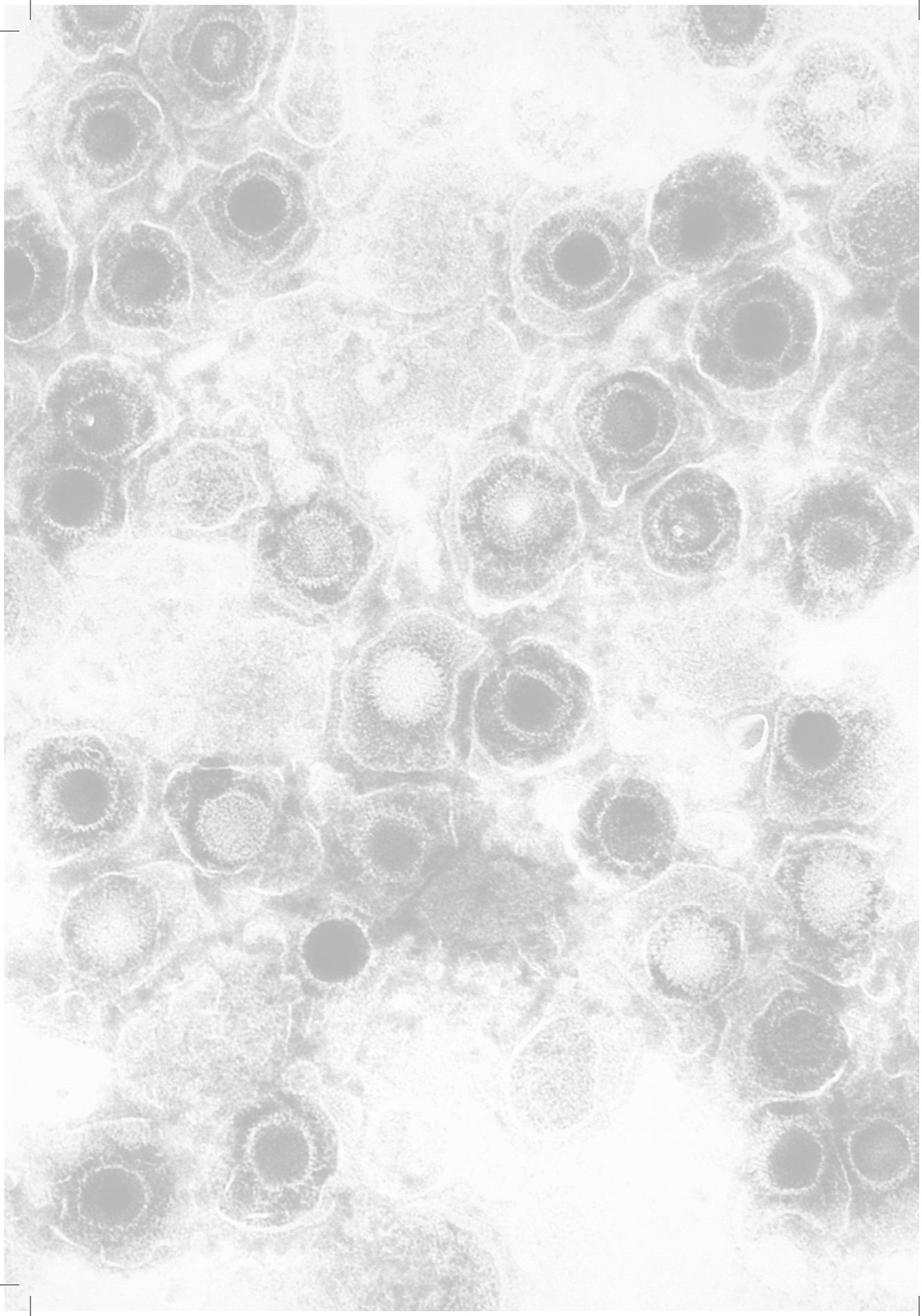

CHAPTER 7

Reduced occipital FA on cerebral diffusion tensor imaging in preterm infants with post-natally acquired cytomegalovirus infection

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Abstract

Background Detection of white matter (WM) abnormalities on MRI is important regarding the neurodevelopmental outcome in preterm infants. The long-term neurodevelopmental outcome of preterm infants with postnatal cytomegalovirus (CMV) infection has not been studied extensively.

Objectives We aimed to assess WM microstructure in preterm infants with postnatal CMV infection using diffusion tensor imaging.

Methods Infants <32 weeks' gestational age (GA) admitted to our hospital between 2007 and 2010, who had cerebral diffusion tensor imaging at term-equivalent age (TEA, i.e. 40 weeks' GA) were included. CMV-PCR in urine collected at TEA was performed to diagnose postnatal CMV infection. Congenital infection was excluded. In the frontal, parietal and occipital WM mean diffusivity, fractional anisotropy (FA), radial and axial diffusivity were calculated. Neurodevelopmental outcome was assessed at 16 months' corrected age using Griffiths' Mental Developmental Scales.

Results Twenty-one postnatally infected and 61 noninfected infants were eligible. Both groups were comparable regarding GA, birth weight and age at MRI. There was a significant difference in median FA of the occipital WM between infected and noninfected infants (0.13 [IQR 0.11–0.16] versus 0.16 [IQR 0.14–0.18], p = 0.002). There were no differences in short-term neurodevelopmental outcome.

Conclusions A significantly reduced FA suggests microstructural changes in the occipital WM of postnatally infected infants. These microstructural changes do not appear to result in impaired neurodevelopmental outcome at 16 months' corrected age.

Introduction

A postnatally acquired cytomegalovirus (CMV) infection is common in preterm infants with a gestational age (GA) <32 weeks.[1-3] The majority of infected preterm infants acquire CMV through breast milk from their seropositive mothers.[1] Although most of the postnatal CMV infections in preterm infants are asymptomatic and may only be detected through screening, sporadically pneumonia, sepsis-like illness or thrombocytopenia may occur.[3] Long-term neurodevelopmental outcome of postnatal CMV infection remains to be established. Postnatally infected preterm infants showed no differences in neurodevelopmental outcome when seen between 2.5 and 4 years of age compared to noninfected controls.[4] However, although still within the normal range, a poorer cognitive and motor function in these infants compared to controls was documented at school age.[5] Congenital CMV infection may lead to the development of cerebral abnormalities and subsequently, impaired neurodevelopmental outcome. We have recently reported that cerebral MRI findings in preterm infants with postnatally acquired CMV infection were very mild compared to congenitally infected infants.[6] To the best of our knowledge, no other studies on cerebral MRI in preterm infants with postnatally acquired CMV infection were reported until now.

White matter (WM) abnormalities in preterm infants demonstrated using MRI at term-equivalent age (TEA, 40 weeks' postmenstrual age) correlates well with adverse neurodevelopmental outcome.[7] Cerebral diffusion tensor imaging (DTI) is an MR modality that determines random diffusion of water in the brain and it is used to detect microstructural alterations in the WM by calculating axial, radial, and mean diffusivity (AD, RD, MD, respectively), and fractional anisotropy (FA).[8]

In this study, we have used axial, radial and mean diffusivity as well as FA at TEA to assess whether postnatally acquired CMV infection in preterm infants affects the structure of the WM.

Methods

Study design

From April 2007 until December 2010, preterm infants with GA <32 weeks admitted to the Neonatal Intensive Care Unit of the University Medical Center Utrecht, The Netherlands were screened routinely at TEA for postnatal CMV infection. MRI of the brain was performed as part of a cohort study between April 2007 and July 2008 as described previously;[9] as part of a prospective cohort study between May 2008 and October 2010,[10] and as part of standard care for preterm infants with GA <28 weeks from October 2010 onwards. One-hundred and thirteen infants with both a known CMV status and a 3-tesla MRI, including high-resolution DTI at TEA were eligible. Subsequently, 13 (12%) infants were excluded because of progressive posthemorrhagic ventricular dilatation (n=6), intraventricular hemorrhage grades III (n=2) and

IV (n=2) according to Papile et al.[11], congenital abnormalities (n=1), cystic periventricular leukomalacia grade III according to de Vries et al.[12] (n=1) and a Gram-negative sepsis (n=1). Demographic, clinical and cerebral ultrasonography (cUS) data were collected as described previously.[2] Informed consent has been obtained and the Internal Review Committee of our hospital approved this study.

Virology

Preterm infants were screened for postnatal CMV infection using CMV-PCR in urine collected at TEA.[6] Congenital infection was excluded using CMV-PCR in urine, collected within 1 week after birth. When urine was not available, highly sensitive CMV-PCR combined with anti-CMV IgM analysis of dried blood spot cards was performed, as described previously.[13]

Magnetic Resonance Imaging

During MRI, infants were sedated with 50–60 mg/kg oral chloral hydrate. MRI was performed on a 3-tesla whole body scanner (Achieva; Philips Healthcare, Best, the Netherlands) using a sense head coil. The MRI contained axial or coronal T1-weighted and T2-weighted images. DTI was based on an axial single-shot EPI sequence with a sense factor of 3 (TE/TR = 48/7745 ms; scan voxel size = 1.42 × 1.44 × 2.0 mm; duration 4.32 min), one b=0 image and a b value of 800 s/mm 2 in 32 directions. All DTIs were registered using registration software of the Philips workstation to correct for eddy currents and rigid motion.

White matter abnormalities on MRI

WM abnormalities on MRI were reviewed by two neonatologists with experience in reviewing MRIs and blinded to the CMV status of the infants. A previously described method[14] based on Woodward et al.[7] was used to assess five areas within the WM including the nature and extent of WM signal abnormality, size of the subarachnoid space, ventricular dilatation, thinning of the corpus callosum and cystic abnormalities. Each area was assigned 1 (normal), 2 (mild abnormality) or 3 (moderate-severe abnormality) points. The scores were summed and the WM categorized as being normal (<7), mildly abnormal (7–9), moderately abnormal (10–12) or severely abnormal (>12).

Diffusion tensor imaging analysis

Analyses and quality assessment of DTI's were performed using the diffusion MRI toolbox ExploreDTI ® (http://www. exploredti.com). Prior to analysis the quality of the DTI was assessed through visual inspection and diffusion tensor estimation by outlier profiles and diffusion tensor residual maps, as described previously by Tournier et al.[15] After analysis, artifacts could be detected through physically implausible FA values (i.e. >1). In the frontal and occipital WM at the level of the basal ganglia, and in the parietal WM at the level of the centrum semiovale, circular regions of interest (ROIs) of 15–18 voxels were drawn in both left and right hemispheres, as described previously[16] by a single investigator. Our intra-observer variation was below 5%.

An example of the ROIs is shown in Figure 1. Subsequently, axial, radial, and mean diffusivity, and FA, were calculated of these ROIs.

Neurodevelopmental outcome

Neurodevelopmental outcome of the infants was assessed routinely using the Griffiths' Mental Developmental Scales (GMDS) at 16 months corrected age.[17]

Statistical analysis

Statistical analysis was performed using PASW statistics ® (version 18.0, SPSS inc., 2009). Dichotomous variables were analyzed using χ^2 test. After analysis of data normality using Kolmogorov-Smirnov test, continuous variables were analyzed using nonparametric Mann-Whitney U test. In demographic and clinical characteristics, p-value< 0.05 was considered statistically significant. All calculated DTI values describe elements of the same microstructural changes, which may provoke type 1 error. To avoid these errors, the p value was divided by 12 (MD, FA, axial and radial diffusivity in three regions) and, therefore, $p<0.004$ was considered statistically significant in MD, FA, axial and radial diffusivity.

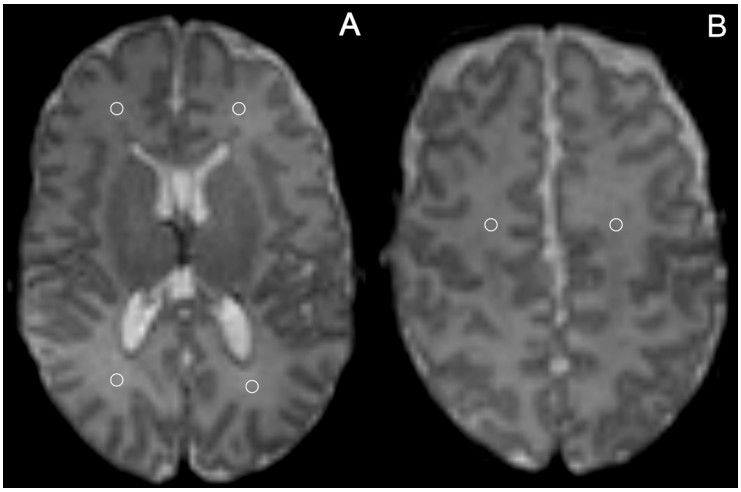

Figure 1. Example of drawn frontal and occipital ROIs [A] and parietal ROIs [B].
Circles represent ROIs of 15-18 voxels.

Results

Study population

After exclusion of 13 infants with severe cerebral abnormalities, DTI measurements were performed in 100 infants, as shown in Figure 2. After DTI quality assessment, including visual inspection, model residual analysis and analysis of implausible FA signals, 18/100 (18%) infants were excluded. Therefore, DTI measurements of 82 infants were analyzed of whom 21 (26%) with postnatal CMV infection. In 19/21 (90%) CMV-positive infants, congenital CMV infection was excluded through CMV-PCR in urine collected within 1 week after birth. In 2 infants (10%), a highly sensitive CMV-PCR combined with anti-CMV IgM analysis of dried blood spot cards had to be used to exclude congenital infection. One of these 21 (5%) infants developed postnatally mild respiratory symptoms due to CMV disease, the other 20/21 (95%) infants were asymptomatic and identified by screening at TEA. Demographic and clinical characteristics of infected and noninfected infants are shown in Table 1.

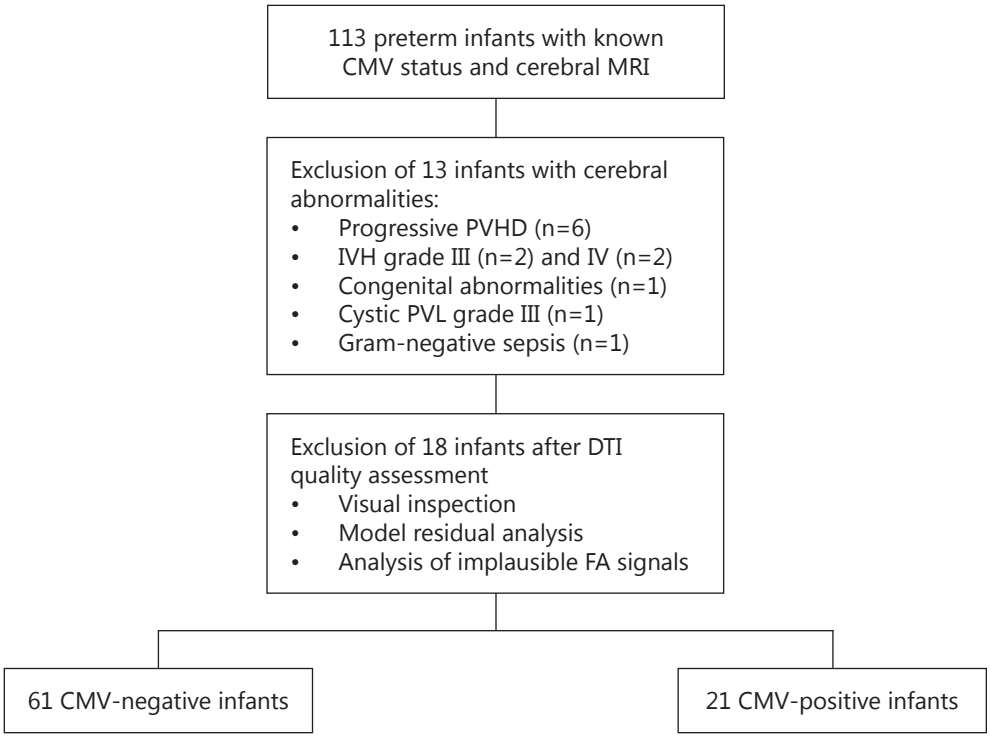

Figure 2. Flow chart.

Table 1. Characteristics of 21 CMV positive infants and 61 CMV negative infants.

	CMV positive infants n=21	**CMV negative infants** n=61	**P value**
Demographic characteristics			
GA, median, weeks (IQR)	26.3 (25.4–27.6)	27.4 (26.1–27.9)	0.186
Age at MRI, median, weeks (IQR)	40.9 (40.4–41.7)	41.0 (40.6–41.7)	0.717
Birth weight, median, grams (IQR)	840 (765–985)	950 (807–1110)	0.170
Small for gestational age	0 (0)	5 (8)	0.197
Non-Dutch maternal ethnicity	9 (43)	8 (13)	**0.004**
Male gender	11 (52)	37 (61)	0.507
Ante- and perinatal characteristics			
Antenatal corticosteroids	16 (76)	53 (87)	0.247
Apgar score at 1min, median (IQR)	5 (4–8)	6 (4–8)	0.906
Apgar score at 5min, median (IQR)	8 (7–9)	8 (7–9)	0.706
Chorioamnionitis*	11 (52)	22 (36)	0.206
Clinical characteristics			
Breast milk	21 (100)	43 (71)	**0.005**
IRDS with use of surfactant	13 (62)	43 (71)	0.466
Mechanical ventilation >7d	5 (24)	15 (25)	0.943
Postnatal corticosteroids	4 (19)	16 (26)	0.509
Chronic lung disease	1 (5)	6 (10)	0.473
PDA, closed with indomethacin	6 (29)	26 (43)	0.255
PDA, closed with surgery	3 (14)	3 (5)	0.155
Use of inotropics	6 (29)	29 (48)	0.130
Sepsis	10 (48)	19 (31)	0.173
Necrotizing enterocolitis	0 (0)	1 (2)	0.555
cUS characteristics			
Intraventricular haemorrhage			
Grade I	2 (10)	12 (20)	0.286
Grade II	4 (19)	4 (7)	0.096
LSV at TEA	8 (38)	7 (11)	**0.007**
Germinolytic cysts at TEA	2 (10)	5 (8)	0.851
cPVL at TEA	0 (0)	0 (0)	1.000

Data are presented as numbers with percentages or mean/medians with interquartile ranges (IQR) in parentheses. * Histopathologic examination of placenta was available in 17/21 (81%) CMV positive infants and 47/61 (77%) CMV negative infants. Abbreviations: cPVL, cystic periventricular leukomalacial; cUS, cerebral ultrasonography; IRDS, infant respiratory distress syndrome; LSV, lenticulostriate vasculopathy; PDA, persistent ductus arteriosus; TEA, term-equivalent age.

White matter abnormalities

The results of the WM abnormality scores are shown in Table 2. There were no significant differences between infected and noninfected infants.

Table 2. Areas of assessment and white matter abnormalities in 21 CMV positive infants and 61 CMV negative infants.

Cerebral white matter abnormalities	**CMV positive infants** n=21	**CMV negative infants** n=61	**P value**
Areas of assessment			
Nature and extent of white matter signal abnormality, median (IQR)	2 (2-2)	2 (2-2)	0.140
Size of the subarachnoid space, median (IQR)	1 (1-2)	1 (1-2)	0.881
Ventricular dilatation, median (IQR)	1 (1-2)	1 (1-2)	0.710
Thinning of the corpus callosum, median (IQR)	2 (1-2)	2 (1-2)	0.148
Cystic abnormalities, median (IQR)	1	1	1.000
Total white matter score			
None	5 (24)	16 (26)	0.827
Mild abnormalities, n (%)	15 (71)	42 (69)	0.825
Moderate abnormalities, n (%)	1 (5)	3 (5)	0.977
Severe abnormalities, n (%)	0 (0)	0 (0)	1.000

IQR: interquartile range

DTI Values

The median axial, radial and mean diffusivity, and FA values between the left and right hemisphere did not vary >10%. Therefore, the averaged values of the left and right hemisphere were used. Results of the frontal, parietal and occipital axial, radial and mean diffusivity and FA analysis in infants with and without postnatal CMV infection are shown in figure 3a–d, respectively. Median axial, radial and mean diffusivity were generally increased and median FA reduced in the frontal, parietal and occipital ROIs of CMV-positive versus CMV-negative infants. However, this difference only reached significance in the FA of the occipital WM (median 0.13 [IQR 0.11–0.16] versus 0.16 [IQR 0.14–0.18], p = 0.002). Analysis of DTI values in CMV negative infants who were fed with breast milk versus CMV-negative infants fed with formula did not yield significant differences (data not shown).

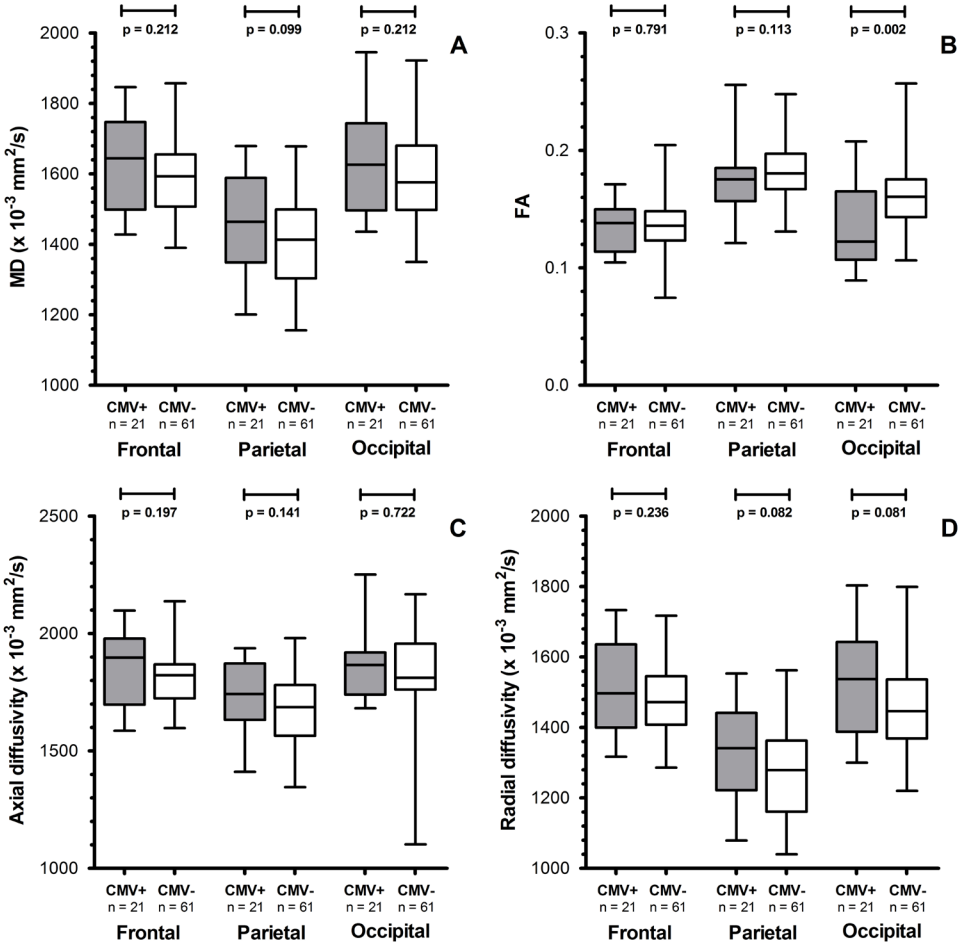

Figure 3. MD (A), FA (B), axial (C) and radial diffusivity (D) in frontal, parietal and occipital WM of infants with (CMV+) and without (CMV-) postnatal CMV infection. Bars in boxplot represent median. Upper and lower limit of boxplot represent 25th and 75th percentile and whiskers represent full range

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Neurodevelopmental outcome

At median 16.1 months (IQR 15.6–16.8), corrected age GMDS was performed in 79/82 (96%) infants, of whom 19 (24%) were infected postnatally. There were no significant differences in global developmental quotient between infected (median 99 [IQR 96–105]) and noninfected infants (median 101 [IQR 94–106]), p = 0.918. Also, there were no differences in the individual scales of GMDS (data not shown).

Discussion

To the best of our knowledge, this is the first report on microstructural changes of the WM in preterm infants with postnatally acquired CMV infection. Microstructural changes, in particular a significantly reduced FA in the occipital WM, were seen in preterm infants with postnatal CMV infection compared to noninfected infants. As more than 90% of seropositive women shed CMV in their breast milk, preterm infants are at risk for CMV infection before they reach TEA.[1,2] Because a premature infant is comparable to a third trimester fetus, one can imagine that CMV infection may result in similar changes as have been reported in the fetus, such as lenticulostriate vasculopathy, germinolytic cysts and signal intensity changes of the WM.[18] The late gestation is crucial for brain maturation and disturbances in key processes or injury to different cell types.[19] Especially immature, premyelinating oligodendrocytes are actively developing and therefore highly vulnerable during the third trimester. While these cells are essential for the myelination process, injury caused by infection, inflammation or ischemia/reperfusion may lead to impaired WM myelination. However, injury to other cells or disturbances of critical events during cerebral development may also result in injury to these oligodendrocytes and subsequently impaired WM myelination.[19] From in vitro animal models and cell culture studies it is known that CMV has the potential to infect most of the central nervous system cells and their progenitors, including oligodendrocytes.[20,21] In the current report, lenticulostriate vasculopathy was more often present in preterm infants with CMV infection compared to noninfected infants, which is in line with our previous research.[2,6] These calcifications clearly seen on cUS are not distinguishable from calcifications found in infants with congenital infection. The significance of these calcifications for neurodevelopmental outcome is uncertain.

In the present study, we have focused on the potential involvement of WM in postnatally acquired CMV infection. cUS and MRI are often used to detect WM injury, with cUS being useful in those with cystic WM injury and MRI in those with subtle WM lesions. As we expected to find microstructural changes in the WM, we performed MRI including DTI at TEA. DTI findings have not been documented previously in preterm infants with postnatally acquired CMV infection. Recently, low FA values on DTI were reported in the parietal/occipital region of three full-term infants with severe congenital CMV infection.[22] These WM changes were comparable with WM changes detected in 4 infants with periventricular leukomalacia suggesting loss of oligodendrocytes within the developing WM. However, neuroimaging was performed at a mean age of 3 years and, therefore data are not comparable with the results of the current study. The clinical relevance of changes found in preterm infants with postnatal CMV infection requires further study.

In gross structural occipital WM injury, symptoms might include impaired development of visual acuity and visual fields.[23] In our study, symptoms related to microstructural changes of occipital WM in particular have not been identified. Furthermore, cerebral visual impairment has not been related to postnatal CMV infection. Studying

microstructural WM abnormalities is important since decreased FA values of DTI in several regions of the premature brain have been associated with impaired neurodevelopmental outcome.[24] A recent small case-control study on the long-term outcome of preterm infants with postnatal CMV infection at a mean age of 8 years showed a poorer cognitive and motor function in infected infants, although performance of both infected and noninfected infants was within the normal range.[5] Unfortunately, neuroimaging was not performed in this study.

In the present study, we show that the neurodevelopmental outcome did not differ between infected and noninfected infants at 16 months corrected age and was within the normal range. Therefore, it seems that subtle microstructural changes in occipital WM do not result in significantly impaired short-term neurodevelopmental outcome. Follow-up assessments are planned. In our study, CMV-infected infants were significantly more often fed with breast milk, a known risk factor of postnatal CMV infection,[1,2] compared to noninfected infants. Use of breast milk as such did not influence our findings, since MD, FA, axial and radial diffusivity in breast-fed infants were similar to those of formula-fed infants in all ROIs.
In line with previous results,[2] postnatal CMV infection occurred more often in infants with a non-native Dutch mother. Only CMV seropositive mothers shed CMV in their breast milk and maternal CMV seropositivity of native Dutch mothers is approximately 50%, in contrast to non-native Dutch mothers from, e.g. Mediterranean or Caribbean countries, in whom seropositivity may reach 100%.[25] There is no consensus on the relation between ethnicity and short- and long-term neurodevelopmental outcome.[26,27] Because of the small study population, we were not able to analyze DTI values with respect to ethnicity.

This study has its limitations. At first, GMDS has no detailed items aiming at the examination of cerebral functions that are localized in the occipital part of the brain. A future study at an age of 5.5 years including cerebral visual function tests will be performed to determine any degree of cerebral visual impairment. A second limitation of this study is its relatively small sample size. Future studies are needed to assess the clinical significance of microstructural WM changes in a large cohort of infants. In conclusion, preterm infants with postnatally acquired CMV infection have significantly reduced FA in the occipital WM compared to non-infected infants. These microstructural changes appear not to be related with impaired neurodevelopmental outcome at 16 months. Further testing at a later age is required to examine occipital white matter integrity.

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CHAPTER 8

Outcome of preterm infants with postnatal cytomegalovirus infection until 6 years of age

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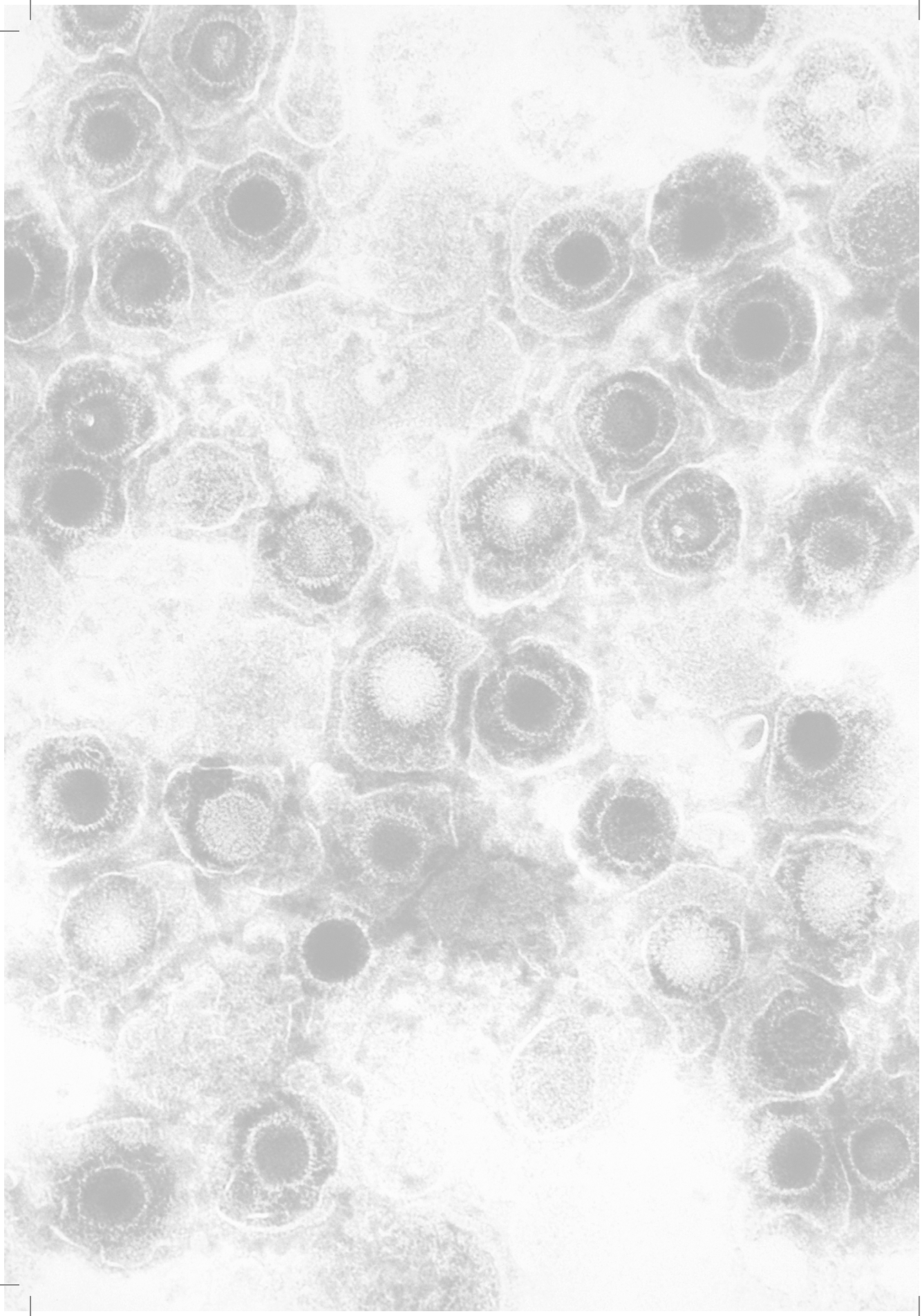

CHAPTER 8

Outcome of preterm infants with postnatal cytomegalovirus infection until 6 years of age

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Abstract

Objective To assess whether preterm infants with postnatal cytomegalovirus (pCMV) infection develop neurological sequelae in early childhood.

Methods Infants with a gestational age <32 weeks were prospectively screened for CMV at term-equivalent age. Neurodevelopment was assessed using the Griffiths Mental Development Scales (GMDS) at 16 months corrected age (CA), the Bayley Scales of Infant and Toddler Development-III (BSITD-III; cognitive and motor scale) at 24-30 months CA, the Movement Assessment Battery for Children-II (MABC-II) and/or the Wechsler Preschool and Primary Scale of Intelligence-III (WPPSI-III) and hearing assessment at 6 years of age.

Results Neurodevelopment was assessed in 356 infants at 16 months CA, of which 49 (14%) were infected and 307 (86%) non-infected. Infected infants performed significantly better on the GMDS locomotor scale. There were no differences at 24-30 months CA on the BSITD-III or GMDS. At 6 years of age, infected children scored lower on the WPPSI-III but the mean scores were within the normal range, reaching significance only on verbal IQ (96 [SD 17] versus 103 [SD15] points; p=0.046) Multiple regression analysis of the subdomains of the WPPSI-III indicated no impact of CMV status but significant influence of maternal education and ethnicity on verbal IQ. There were no significant differences between both groups on the MABC-II. None of the infected children developed perceptive hearing loss at 6 years of age.

Conclusion In this cohort study, pCMV infection in preterm born children did not have an adverse effect on neurodevelopmental outcome within the first five-six years of life.

Introduction

Postnatal cytomegalovirus (pCMV) infection is a common viral infection and frequently affects preterm infants (gestational age [GA] <32 weeks) and very low birthweight (VLBW; birthweight <1500 grams) infants, with an estimated median incidence of 20%.[1,2] CMV is mainly transmitted via CMV-seropositive mothers shedding the virus in their breastmilk, of which around 37-76% will transmit the virus to their infants.[3,4] While term infants are asymptomatic, preterm infants and VLBW infants may be at risk for symptomatic disease.[5,6] Symptomatic disease such as CMV sepsis-like syndrome, thrombocytopenia, pneumonia and/or hepatitis may occur, but is rare (median incidence 3.6%).[1,5,7] CMV is the leading non-genetic cause of sensorineural hearing loss (SNHL) amongst infants with congenital CMV infection.[8] SNHL was not found in infants with pCMV infection at two years[9] and eight years of age.[10] Data on neurodevelopmental outcome of children with pCMV infection are scarce and limited to small cohort studies. In preterm infants, neurodevelopment until four years of age appears to be within the normal range.[10–12] Several studies however have suggested a negative impact on cognitive development at school age.[13–15] Because of the uncertainty regarding the short- and long-term consequences of a pCMV infection, fresh breast milk is not always recommended for VLBW infants.[16,17] Since (untreated) breast milk is known to improve infant health,[18] it is important to study the effects of pCMV infection in a large cohort of preterm infants. The aim of this prospective, longitudinal cohort study is to examine the consequences of a pCMV infection on neurodevelopmental outcome including hearing in a cohort of preterm infants until 6 years of age.

Methods

Study population

From April 2007 until December 2010, all preterm infants (GA<32 weeks) admitted to the level three Neonatal Intensive Care Unit (NICU) of the Wilhelmina's Children Hospital, Utrecht, The Netherlands were screened for CMV, predominantly in urine obtained at term-equivalent age (TEA; 40 weeks postconceptional age) using CMV polymerase chain reaction (PCR) as previously described.[6] Congenital CMV infection was excluded as previously described.[19] Exclusion criteria were: absence of urine at TEA, severe cerebral abnormalities (i.e. porencephalic cyst, cystic periventricular leukomalacia, post-hemorrhagic ventricular dilatation requiring insertion of a ventricular reservoir or ventriculo-peritoneal shunt, intraventricular hemorrhage grade III and IV), chromosomal anomalies, death before TEA, and no parental consent. Infants were exclusively fed with fresh breast milk from their own mothers. For the analysis of neurodevelopmental outcome at 6 years, only children with a GA≤30 weeks were included. The Internal Review Committee of our hospital approved this study.

Case definitions

Clinical and demographic characteristics were collected as previously described.[6] Additionally, small for gestational age (SGA), socio-economic status (SES) and maternal education were recorded. SGA was defined as birthweight by GA<10th percentile. Percentiles for our population were obtained from the Dutch perinatal registry.[20] SES was determined indirectly using the SES of the parent's zip code, and was provided by The Netherlands Institute for Social Research.[21] A score below -1 was considered low, between -1 and 1 average, and above 1 high. Symptoms of pCMV-disease included sepsis-like illness, pneumonia, cholestasis and/or thrombocytopenia.[5] Criteria for diagnosis of symptomatic CMV-disease have been described previously.[6] During admission to the NICU and at TEA, cranial ultrasonography was performed as previously described.[6]

Neurodevelopmental assessment

Neurodevelopmental outcome was routinely assessed by developmental specialists at the outpatient clinic using the Griffiths Mental Development Scales (GMDS)[22] at 16 months corrected age (CA) and the Bayley Scales of Infant and Toddler Development-III (BSITD-III)[23] at 24–30 months CA. The BSITD-III is routinely used in infants with a GA<30 weeks at 24–30 months. When infants had a higher GA (GA 30–32 weeks) the GMDS was used instead. At 6 years of age, motor function was routinely assessed in all preterm born children using the Movement Assessment Battery for Children-II (MABC-II).[24] Cognitive function was routinely assessed at our hospital using the Wechsler Preschool and Primary Scale of Intelligence-III (WPPSI-III)[25] in all children born with a GA≤28 weeks. Children born with a GA>28 weeks who have abnormal development at 6 years are only tested with the WPPSI-III at the request of the pediatrician. Therefore inclusion numbers are lower for the WPPSI-III than the MABC-II (Figure 1). All eligible infants with a pCMV infection and a GA>28 weeks were tested using the WPPSI-III as part of this study. A detailed account of the GMDS, the BSITD-III, the MABC-II and the WPPSI-III has been previously described.[26,27] Z-scores were calculated for the GMDS and BSITD-III to compare outcome at two years of age. Both GMDS and BSITD-III scores were corrected for preterm birth. When BSITD-III was administered at 24 months CA, parents were asked to provide the age of onset of independent walking (AOIW) defined as walking at least five steps independently.[23]

Hearing assessment

At 6 years of age, all children with a pCMV infection underwent pure tone audiometric testing with headphones, following standardized procedures in a tertiary care level audiology center.

When pure tone audiometric testing was not feasible due to patient incompliance, behavioral observation audiometry was used instead. Pure tone averaged (500, 1000, 2000, 4000 Hz) hearing loss was calculated for each ear. Audiograms were assessed for conductive or sensorineural hearing loss. Speech performance curves were measured using the standardized Dutch CVC-list for children[28] and assessed for maximum speech discrimination score and curve displacement (>20 dB from

reference curve). Middle ear function was assessed by impedance audiometry. The degree of hearing impairment was classified according to the World Health Organization grading system.[29] SNHL was defined as a threshold elevation of >25 dB without any component of conductive hearing loss.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics version 23. Figures were produced in GraphPad Prism® (version 5.03, GraphPad Software, Inc., La Jolla USA, 2009). Categorical and dichotomous variables were analyzed using chi-square test. Continuous variables were analyzed with two-tailed student's T test. One-way ANOVA was used to determine correlations between continuous variables. A p-value of <0.05 was considered statistically significant. Multiple regression analyses were performed with AOIW and with the subsets of the WPPSI-III, as dependent variables and pCMV infection and significantly correlated parameters as independent variables.

Results

Study population

During the study period, CMV status at TEA could be determined in 462/701 (66%) preterm infants with a GA<32 weeks, of which 411/462 (89%) were eligible for follow-up (Figure 1). CMV status could not be determined in 186/701 (27%) infants because collection of urine at TEA was not successful. A pCMV infection was diagnosed in 74 infants, of which congenital CMV infection was excluded in 63/74 (85%) infants using CMV-PCR of the urine collected in the first week and in 11/74 (15%) infants using CMV-PCR combined with anti-CMV IgM analysis of dried blood spots cards. Of the infected infants 56/74 (76%) could be included in the hearing study. Clinical symptoms of CMV-disease were observed in 4/74 (5%) infants and included thrombocytopenia (n=1), pneumonia (n=2) and sepsis-like-illness with pneumonia and thrombocytopenia (n=1). One infant with symptomatic pCMV infection died at six months of age due to respiratory problems not associated with CMV. None of the infants were treated with (val)ganciclovir. All CMV positive infants were fed fresh breast milk from their mothers. Clinical and demographic data with respect to CMV status are summarized in Table 1 for infants tested at 24-30 months and for children tested at 6 years of age. The clinical characteristics of infants assessed at 16 months were comparable with infants assessed at 24-30 months (data not shown). Baseline characteristics of the infants tested with the WPPSI-III at 6 years of age can be found in Table S1 (Supplemental data). Baseline characteristics of the children receiving hearing assessment at 6 years of age can be found in Table S2 (Supplemental data). Overall, infected infants were significantly more often born to mothers of non-Western origin, were fed fresh breast milk more frequently and had LSV at TEA more frequently (Table 1), findings that have been previously reported.[6]

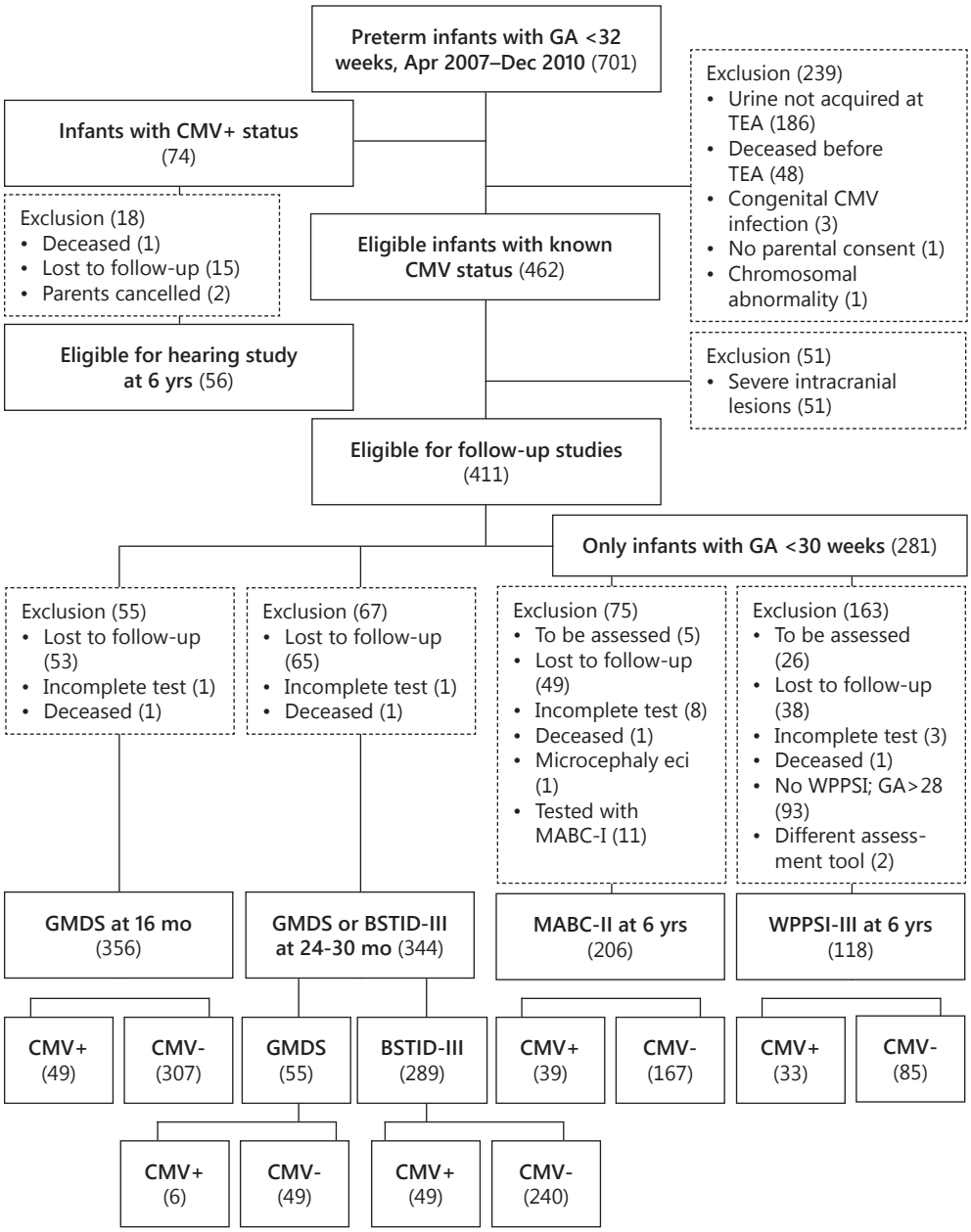

Figure 2. Inclusion of study population. Numbers in brackets. GMDS, Griffiths mental developmental scales; BSITD-III, Bayley scales of infant and toddler development-III; MABC-II, Movement Assessment Battery for Children-II; WPPSI-III, Wechsler Preschool and Primary Scale of Intelligence-III; GA, gestational age.

Table 1. Clinical and demographic characteristics of preterm infants assessed with BSITD-III or GMDS at 24 months CA and/or at 6 years of age with the WPPSI-III and/ or the MABC-II with respect to postnatal CMV infection.

	Infants assessed at 24m CA (GA <32 wk)			Infants assessed at 6y (GA≤30 wk) with WPPSI-III and/or MABC-II		
	CMV+ n=55	CMV- n=289	P	CMV+ n=41	CMV- n=172	P
Patient characteristics						
GA, mean, wk (SD)	28.2 (2)	28.8 (2)	**0.022**	27.7 (1.5)	28 (1)	0.242
Birthweight, mean, g (SD)	1129 (287)	1181 (319)	0.261	1076 (266)	1074 (257)	0.964
SGA	5 (13)	39 (14)	0.803	1 (2)	16 (9)	0.145
Male gender	31 (56)	157 (54)	0.781	23 (56)	83 (48)	0.367
NWMO*	30 (55)	42 (15)	**<0.001**	25 (61)	21 (12)	**<0.001**
Apgar at 1 min, mean (SD)	7 (2)	7 (2)	0.231	7 (2)	6 (2)	0.463
Apgar at 5 min, mean (SD)	8 (2)	8 (1)	0.592	8 (1)	8 (1)	0.965
Breastmilk	55 (100)	229 (79)	**<0.001**	41 (100)	137 (80)	**0.002**
RDS	22 (40)	150 (52)	0.106	19 (46)	112 (65)	**0.026**
>1w on ventilator	3 (6)	45 (16)	**0.047**	2 (5)	31 (18)	**0.037**
Chronic lung disease	1 (2)	16 (6)	0.244	1 (2)	14 (8)	0.200
PDA	12 (22)	71 (25)	0.662	10 (24)	51 (30)	0.503
Surgery for PDA	2 (4)	10 (4)	0.948	2 (5)	7 (4)	0.817
Hypotension (inotropes)	11 (20)	105 (36)	**0.019**	6 (15)	72 (42)	**<0.001**
Transfusions, median (range)	1 (0–15)	1 (0–18)	0.881	2 (0–15)	2 (0–18)	0.598
Sepsis	13 (24)	94 (33)	0.192	8 (20)	59 (34)	0.067
NEC	0	9 (3)	0.185	0	6 (4)	0.225
NICU, days, mean (SD)	33 (21)	34 (24)	0.890	38 (21)	41 (24)	0.435
Socio-economic status						
Low	12 (22)	35 (12)	0.055	11 (27)	19 (11)	**0.009**
Average	34 (62)	192 (66)	0.508	23 (56)	116 (67)	0.170
High	9 (16)	62 (22)	0.393	7 (17)	37 (22)	0.528
Cranial ultrasonography findings						
IVH						
Grade I	5 (11)	29 (14)	0.576	5 (12)	20 (12)	0.919
Grade II	6 (13)	27 (13)	0.996	4 (10)	24 (14)	0.475
LSV	20 (36)	49 (17)	**0.001**	15 (37)	34 (20)	**0.021**
Germinolytic cysts*	5 (15)	12 (13)	0.729	5 (12)	25 (15)	0.699

Data are presented as numbers with percentages or mean/medians with ranges in parentheses. GMDS: Griffiths Mental Development Scales; BSITD-III: Bayley scales of infant and toddler development-III; WPPSI-III: Wechsler Preschool and Primary Scale of Intelligence-III; MABC-II: Movement Assessment Battery for Children-II; CA: corrected age; GA: gestational age; wk: weeks; n: number; SD: standard deviation; SGA: small for gestational age; PDA: patent ductus arteriosus; NICU: neonatal intensive care unit; IVH: intraventricular hemorrhage; LSV: lenticulostriate vasculopathy; TEA: term equivalent age; RDS: respiratory distress syndrome; NEC: necrotizing enterocolitis; NWMO: non-western maternal origin. * at term equivalent age.

GMDS at 16 months

A total of 49/356 (14%) infants with a pCMV infection and 307/356 (86%) non-infected infants were tested at a mean age of 16.4 months CA (SD 1.8) and 16.1 months CA (SD 1.3), respectively. The mean locomotor subscale quotient was significantly higher in infants with pCMV infection (Z-score 0.35 [SD 0.81] versus 0.02 [SD 0.98] in the control group which corresponds to a quotient of 102 [SD 12] versus 97 [SD 14], p=0.025) (Figure 2A). Other subscales did not differ significantly. The mean general DQ was comparable in infected and non-infected infants (102 [SD 9] versus 101 [SD 9], p=0.320, respectively) and was within the normal range.

GMDS or BSITD-III at 24 to 30 months

A total of 49/289 (17%) infants with a pCMV infection and 240/289 (83%) non-infected infants were tested using the BSITD-III at a mean of 26.1 months CA (SD 3.0) and 25.3 months CA (SD 2.5), respectively. In 6/55 (11%) infected infants and 49/55 (89%) non-infected infants the GMDS was used instead at a mean age of 23.1 months (SD 1.2) and 24.6 months (SD 1.7), respectively. There were no significant differences in BSITD-III and GMDS Z-scores between infected and non-infected infants (Figure 2B and 2C, respectively). The mean corrected cognitive composite scores and total motor composite score in infected and non-infected infants using the BSITD-III at 24 months (104 [SD 10] versus 105 [SD 12], p=0.184; 109 [SD 10] versus 109 [SD 12] p=0.748, respectively) were within the normal range.

Age of independent walking

Mean AOIW was compared between 49 infants with pCMV infection and 239 non-infected infants. Postnatally infected infants were able to walk at a younger CA compared to non-infected infants (14.7 months [SD 2.4] and 15.8 months [SD 3.1], p=0.026, respectively). Multiple regression analysis showed that significantly earlier AOIW was related to non-Western maternal ethnicity (Table S3, supplemental data). Mean AOIW was 14.1 months [SD 2.8] in infants of mothers of non-Western ethnicity compared to mean AOIW of 16.0 months [SD 3.0] in infants of mothers of Western ethnicity (p<0.001).

WPPSI-III at 6 years of age

In total, 33/118 (28%) children with a pCMV infection and 85/118 (72%) non-infected children were tested at a mean age of 5.7 years (SD 0.3) and 5.7 years (SD 0.5), respectively. Mean scores were in the normal range on all four domains for infected and non-infected children, but infected children had overall lower scores (Table 2A). This only reached statistical significance on the subscale of verbal IQ (96 [95%CI 90–102] points versus 103 points [95%CI 100–106], p=0.046). Multiple regression analyses indicated that the presence of RDS significantly impacted total IQ (coefficient -5.9, p=0.046) and verbal IQ (coefficient -8.8, p=0.004) (Table S4, supplemental data). Non-Western maternal origin (coefficient -7.7, p=0.049), and low maternal education (coefficient -8.3, p=0.019) also both significantly impacted verbal IQ.

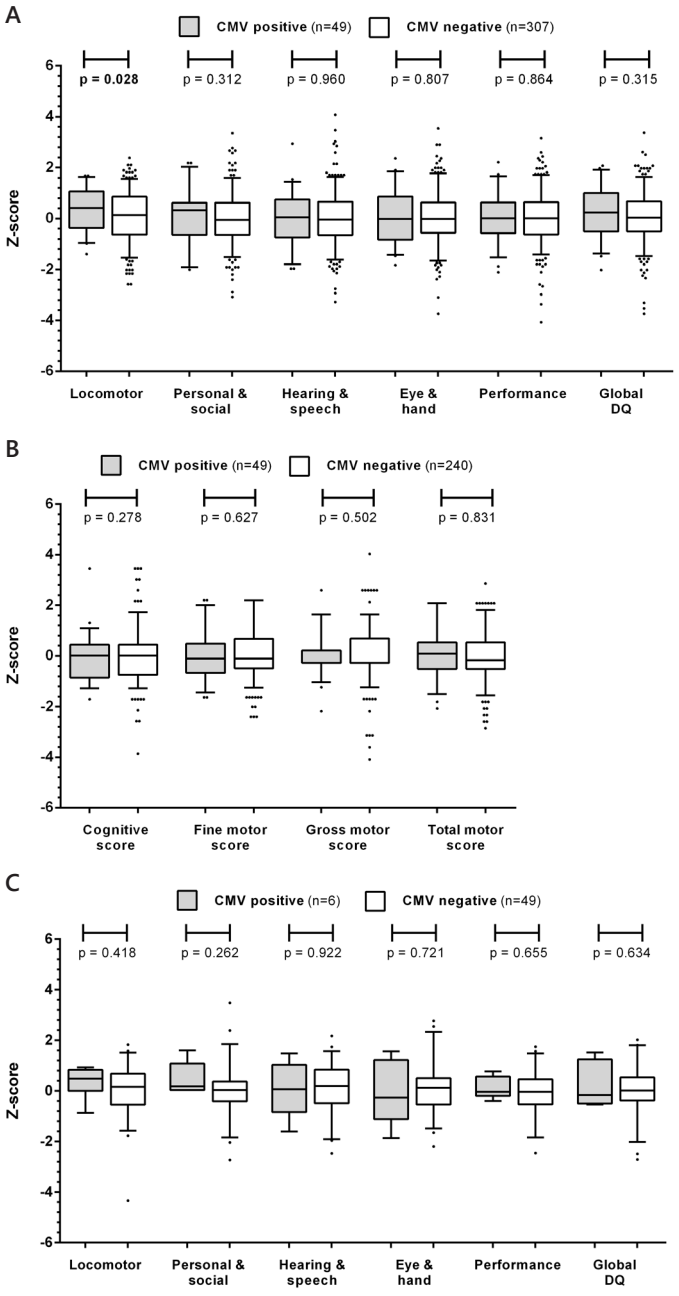

Figure 2. Z-scores of [A] GMDS at 16 months CA, [B] BSITD-III at 24 months CA, [C] GMDS at 30 months CA. Upper and lower borders of box plots represent the 25th and 75th percentile, bars represent median Z-scores and whiskers represent 5th and 95th percentile. Outliers are depicted as dots. DQ – developmental quotient.

Table 2. Results of the WPPSI-III and of the MABC-II at 6 years of age.

	CMV positive infants n=33	**CMV negative infants** n=85	**P**
WPPSI-III domains			
Total IQ, mean , 95% CI[1]	96 (91–101)	101* (98–104)	0.079
Verbal IQ, mean, 95% CI	96 (90–102)	103 (100–106)	**0.046**
Performance IQ, mean, 95% CI	100 (95–105)	102 (99–105)	0.456
Processing speed Quotient, mean, 95% CI[2]	92Δ (86–98)	93□ (90–96)	0.708
	CMV positive infants n=39	**CMV negative infants** n=167	**P value**
MABC-II standard score			
Total impairment score, median, range[3]	8 (4–14)	9 (2–15)	0.661
Manual dexterity, median, range	8 (4–14)	9 (2–15)	0.902
Aiming & catching (Ball skills), median, range	10 (5–17)	9 (1–15)	0.052
Dynamic and static balance, median, range[4]	8 (4–16)	9 (2–17)	0.081

WPPSI-III: Wechsler Preschool and Primary Scale of Intelligence-III; MABC-II: Movement Assessment Battery for Children-II; 1: data from one child missing; 2: data from six children missing; 3: data from one child missing; 4: data from one child missing.

CMV was not significantly associated with impaired outcome on any of the four WPPSI-III domains. No significant differences were found between symptomatic and asymptomatic children (data not shown).

MABC-II at 6 years of age

Thirty-nine (19%) children with a pCMV infection and 167 (81%) non-infected children were tested at a mean age of 5.8 years (SD 0.2) and 5.7 years (SD 0.4), respectively (p=0.155). There were no significant differences in median scores of the MABC-II subscales between both groups (Table 2B). The median total impairment standard score between infected children and non-infected children was eight points and nine points (p=0.661), respectively and was in the normal range.

Hearing outcome at 6 years of age in children with pCMV infection

Seventy-four (16%) children had a pCMV infection of which 56 (76%) were audiologically tested at a mean age of 5.8 years of age (SD 0.2) (Figure 1). None of the children had SNHL. Pure tone audiometry was carried out in all children. Forty-four (79%) children had normal hearing. A slight hearing impairment (26-40 dB) was seen in nine (16%) children of whom seven had unilateral conductive hearing loss and two

had bilateral conductive hearing loss. Tympanometry indicated middle ear dysfunction in all (middle ear fluid, n=2; negative pressure in middle ear cavity, n=5; middle ear fluid & negative pressure in middle ear cavity, n=2). A moderate impairment (41-60 dB) was seen in 3 (5%) children of which one had unilateral conductive hearing loss and two had bilateral conductive hearing loss. Again tympanometry indicated middle ear dysfunction in all children (middle ear fluid n=1; negative pressure in middle ear space n=1; cholesteatoma and negative pressure in middle ear space n=1). Speech audiometry was carried out in two and reflected the conductive moderate impairment seen on pure tone audiometry. Cochlear function was normal in all tested children.

Discussion

To the best of our knowledge, this is the largest prospective cohort study examining neurodevelopmental outcome of preterm infants with a pCMV infection from birth until early childhood. The results of this study did not show an impaired neurodevelopmental outcome (including SNHL) until 6 years of age/early childhood in preterm born infants with pCMV infection. So far, outcome studies have given an inconclusive result of the long-term effects of a pCMV infection in preterm infants. While short term development until 2–4.5 years of age seems to be unaffected[11,12,30] more recently, smaller case-controlled studies following infected children until school age and into adolescence have shown subtle impairments in cognitive functioning when compared to uninfected controls.[10,13,14,31]

In line with previous studies on short-term outcome,[11,12,30] we did not find any adverse neurodevelopmental sequelae in 55 preterm infants with pCMV infection until two years CA compared to 289 non-infected infants. Cognitive assessment at 6 years of age/in early childhood indicated slight discrepancies between the groups. Infected children overall scored in the normal range but had lower mean scores on all domains compared to uninfected children, reaching significance on the domain of verbal IQ only. CMV status did not significantly contribute to the observed variance on any subscales of the WPPSI-III. Maternal non-Western ethnicity and low maternal education were significantly associated with a lower verbal IQ score. Non-Western maternal ethnicity has been previously identified as a risk factor for postnatal CMV acquisition[6] and children with pCMV infection in this study had significantly more mothers of non-Western ethnicity. It is conceivable that children, whose parents do not speak Dutch as their first language, may learn the native language of their parents first. As such, the WPPSI-III which is conducted in Dutch at our institution, may pose a significant language barrier to the child.

A similar trend was also observed in a study by Bevot et al. in which 20 children with pCMV infection and 21 non-infected controls were assessed using the Kaufmann Assessment Battery (KABC) and the MABC-I at eight years of age.[32] Cognitive scores were in the normal range; however infected children scored significantly lower on

the cognitive composite score and simultaneous processing score. Contrarily, pCMV infection contributed independently to lower scores on all cognitive subscales and in combination with paternal SES on the overall cognitive composite score. The strong effect of parental education was confirmed in this study, however the independent contribution of CMV status on the lower test scores was not found.

In a subsequent larger study, 42 children with pCMV infection and 42 controls were examined using the Kaufmann Assessment Battery for Children at six years of age.[13] This study included the same cohort as Bevot et al., as well additional infants born at a lower GA (GA<30 weeks and birthweight <1000 grams). Again, all infected children scored in the normal range but lower than the controls. Interestingly, with the expansion of the study cohort the significant differences initially observed by Bevot et al with the smaller group, were not confirmed. Similar to our study, lower SES (consisting also of parental education) significantly contributed to the lower scores. The authors however, did not examine the absolute contribution of CMV status on outcome, but rather showed that infants infected before discharge scored significantly lower than infants infected after discharge. It would have been of interest to examine the scores of infants who acquired CMV before discharge, stratified for the GA of CMV acquisition.

In the most recent study by Brecht et al., the earlier studied cohort now at 11–17 years of age had significantly lower general intelligence scores than non-infected preterm and term adolescents, findings that could not be explained by differences in maternal education, attention or brain pathology.[14] The authors suggested that the effects of an early pCMV infection may only manifest at school age/adolescence at a time when complex, higher cognitive functions develop.[14] An important confounder not controlled for in this study is parental ethnicity. Lower maternal education and immigrant background of both parents have been linked to an impaired long-term composite intelligence quotient at 10-13 years of age.[33]
A recent study observed an increased occurrence of bronchopulmonary dysplasia in VLBW infants with pCMV infection.[34] Due to the chosen inclusion methods, selection bias may have been introduced and most probably infants with congenital CMV infection have been included, therefore these results should be interpreted with caution.

In the current study, development of bronchopulmonary dysplasia was similar in both groups. In our population studied at 6 years, the control group had significantly more RDS and mechanical ventilation for >7 days. RDS independently contributed to a lower total IQ and verbal IQ and but still the control group attained a higher score compared to infected infants. It is possible that the differences in scores between both groups may have been more pronounced without baseline differences in neonatal morbidity (including RDS).

In terms of motor development, infected infants had a better gross motor performance at 16 months CA and had a significantly earlier AOIW. While the majority of infected infants were non-Western, mostly of Turkish and Moroccan ethnicity, we analyzed the association between pCMV infection and AOIW for possible confounders and found that non-Western maternal ethnicity had a positive effect on independent walking. Ethnicity has previously been associated with AOIW in a study on AOIW in Dutch preterm infants.[35] To the best of our knowledge these findings have not been previously documented amongst infants with pCMV infection. At 6 years of age all median scores were in the normal range without significant differences on all subscales of the MABC-II. In the study by Bevot et al. infected children at eight years of age scored lowest on the visual processing scale of the KABC and on the subscale of ball skills of the MABC-I, which focuses on hand-eye coordination.[32] We have previously demonstrated microstructural changes in occipital white matter of infants with pCMV infection,[36] however using the MABC-II at 6 years of age, infected children actually scored better than non-infected children on ball skills. The significance of these microstructural changes remains to be elucidated. Due to the use of the MABC-II in our analysis however, these results are not comparable, as normative values for scoring and test execution differ.

Previously and in this study, we have shown that lenticulostriate vasculopathy not yet present at birth is more common in preterm infants with pCMV infection.[6] This neuro-imaging finding is not associated with impaired neurodevelopmental outcome at two years CA and at 6 years of age.

SNHL is a well-known sequela of congenital CMV infection[8] but has so far not been detected in infants with pCMV infection.[5] At 12 months and 24 months SNHL has been previously excluded for this cohort[37] and at 6 years of age none of the infected infants still included in the study developed SNHL.

This study has several limitations, most importantly that we did not determine the onset of CMV infection and therefore we cannot exclude that an early pCMV infection may still have detrimental effects on neurodevelopment. Timing of CMV acquisition and subsequent first viral detection, in relation to birthweight and GA seem to be the most important risk factors for symptomatic CMV-disease.[7,38,39] In this study, of the infants with a pCMV infection assessed at 5–6 years of age (n=33), 9% (n=3) were symptomatic and no differences in outcome were noted (data not shown). Furthermore, there was the common issue of loss-to-follow up in long-term prospective follow-up studies, which may have introduced selection bias. More prospective cohort studies are needed whereby the timing of virus acquisition and symptomatic disease are correlated to long-term neurodevelopmental outcome.

The results of the present study do not justify interventions like pasteurization, freezing or withholding breast milk to prevent CMV transmission. Positive effects of breast milk[40] are still likely to outweigh the possible adverse effects of a pCMV infection. In extremely preterm and VLBW infants however, CMV may act as an aggravator of an already fragile system causing symptomatic infection.[5,41] Infants in these cases may benefit from a delayed introduction of breast milk or pre-treatment of breast milk to prevent symptomatic pCMV infection[5] until the exact long-term consequences are determined.

In conclusion, neurodevelopmental outcome in children with pCMV infection is normal in early childhood. At 6 years of age, infected children had lower cognitive scores, with only a significant difference for the verbal IQ, which could be attributed to maternal education and ethnicity and not CMV status. Median motor function at 6 years of age was within the normal range. None of the infected children developed perceptive hearing loss. More prospective cohort studies are needed to examine cognitive development in extreme preterm infants (GA<28 weeks) to determine the consequences of early and/or symptomatic pCMV infection.

Supplemental data

Table S1. Baseline characteristics of children tested with the WPPSI-III at 6 years of age.

	WPPSI-III only infants (GA≤30 weeks)		
	CMV+ n=33	CMV- n=85	P
Clinical and demographic characteristics			
Gestational age, mean, wk (SD)	27.6 (1.4)	27.4 (1.2)	0.356
Birthweight, mean, g (SD)	1074 (273)	945 (209)	**0.018**
SGA	1 (3)	12 (14)	0.084
Male gender	21 (64)	45 (53)	0.294
Non-western maternal origin	20 (61)	7(8)	**<0.001**
Apgar at 1 min, mean (SD)	7 (2)	6 (2)	0.122
Apgar at 5 min, mean (SD)	8 (1)	8 (1)	0.748
Breastmilk	33 (100)	65 (77)	**0.002**
RDS	13 (39)	66 (77)	**<0.001**
>7 days of mechanical ventilation	2 (6)	26 (31)	**0.005**
Chronic lung disease	1 (3)	8 (9)	0.241
PDA	9 (27)	31 (37)	0.343
Surgery for PDA	2 (6)	4 (5)	0.764
Hypotension treated with inotropes	4 (12)	48 (57)	**<0.001**
Number of transfusion, median (range)	2 (0–15)	4 (0–18)	**0.015**
Sepsis	5 (15)	36 (42)	**0.005**
Necrotizing enterocolitis	0	2 (2)	0.374
NICU admission days, mean (SD)	36 (20)	52 (22)	**<0.001**
Socio-economic status			
Low	10 (30)	12 (14)	**0.043**
Average	17 (52)	56 (66)	0.149
High	6 (18)	17 (20)	0.823
Cranial ultrasonography findings			
IVH			
Grade I	5 (11)	29 (14)	0.576
Grade II	6 (13)	27 (13)	0.99w6
LSV at TEA	20 (36)	49 (17)	**0.001**
Germinolytic cysts at TEA	5 (15)	12 (13)	0.729
Maternal education			
Low	4 (12)	18 (21)	0.257
Average	11 (33)	28 (33)	0.968
High	17 (52)	39 (46)	0.582

Data are presented as numbers with percentages or mean/medians with ranges in parentheses. WPPSI-III: Wechsler Preschool and Primary Scale of Intelligence-III; GA: gestational age; wk: weeks; n: number; SD: standard deviation; SGA: small for gestational age; RDS: respiratory distress syndrome; PDA: patent ductus arteriosus; NICU: neonatal intensive care unit; IVH: intraventricular hemorrhage; LSV: lenticulostriate vasculopathy; TEA: term equivalent age.

Table S2. Baseline characteristics of children with pCMV infection and hearing assessment at 6 years of age.

	Hearing test at 6 years of age n=56
Gestational age, mean, wk (SD)	27.9 (1.8)
Gestational age ≤27 wk	21 (38)
Birthweight, mean, g (SD)	1070 (263)
Male gender	31 (55)
Symptomatic pCMV infection	3 (5)
LSV at TEA	20 (36)
Germinolytic cysts at TEA	6 (15)
IVH present on cUS	18 (32)
Grade I,	5 (9)
Grade II	8 (14)
Grade III	3 (5)
Grade IV	2 (4)
Treated PHDV	5 (26)
CMV load, median, IU/ml (range)	$2.8x\ 10^5$ ($1.3x\ 10^2$ – $3x\ 10^7$)
>7 days of mechanical ventilation	7 (13)
HFO ventilation	6 (11)
PDA treated with indomethacin	17 (30)
Hypotension treated with inotropes	11 (20)
Use of loop diuretics	5 (9)
Use of aminoglycosides	54 (96)

Data are presented as numbers with percentages or mean/medians with ranges in parentheses. Wk: weeks; n: number; SD: standard deviation; IVH: intraventricular hemorrhage; LSV: lenticulostriate vasculopathy; TEA: term equivalent age; PHVD: post-hemorrhagic ventricular dilatation; HFO: High-frequency oscillation.

Table S3. Multiple regression analysis of AOIW.

	AOIW		
R^2	**B**	**95% CI**	**P**
0.072			
NWMO	-1.7	-2.7 – -0.8	**0.001**
MV >7 days	0.9	-1.4 – 0.3	0.060
CMV	-0.2	-1.1 – 0.9	0.744

AIOW: age of independent walking; NWMO: Non-Western maternal origin; MV >7 days: mechanical ventilation >7 days; CMV: cytomegalovirus.

Table S4. Multiple regression analysis of the four domains of the WPPSI-III.

	TIQ			VIQ			
	R^2	B	95% CI	P value	R^2	B	95% CI	P value
	0.10				0.18			
NWMO		-5.6	-13 – 1.8	0.136		-7.7	-15.3 – -0.04	0.049
RDS		-5.9	-11.6 – -0.1	0.046		-8.8	-14.8 – -2.9	0.004
LME		-5.4	-12 – 1.3	0.111		-8.3	-15.1 – -1.4	0.019
CMV		-5	-12.2 – 2.3	0.178		-6.4	-13.9 – 1.1	0.093
	PIQ				PS			
	R^2	B	95% CI	P value	R^2	B	95% CI	P value
	0.04				0.04			
NWMO		-4.7	-11.8 – 2.3	0.186		7.4	-0.04 – 14.9	0.051
RDS		-3.7	-9.2 – 1.8	0.187		-0.3	-6.2 – 5.5	0.914
LME		-0.3	-6.6 – 6.1	0.936		-4.3	-11.0 – 2.4	0.209
CMV		-1	-7.9 – 5.9	0.779		-5.5	-12.8 – 1.9	0.142

WPPSI-III: Wechsler Preschool and Primary Scale of Intelligence-III; B: unstandardized coefficient; 95%CI: 95% confidence intervals; TIQ: Total IQ; VIQ: Verbal IQ; PIQ: Performance IQ; PS: Processing speed Quotient; NWMO: Non-Western maternal origin; RDS: respiratory distress; LME: low maternal education; CMV: cytomegalovirus.

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Founding source

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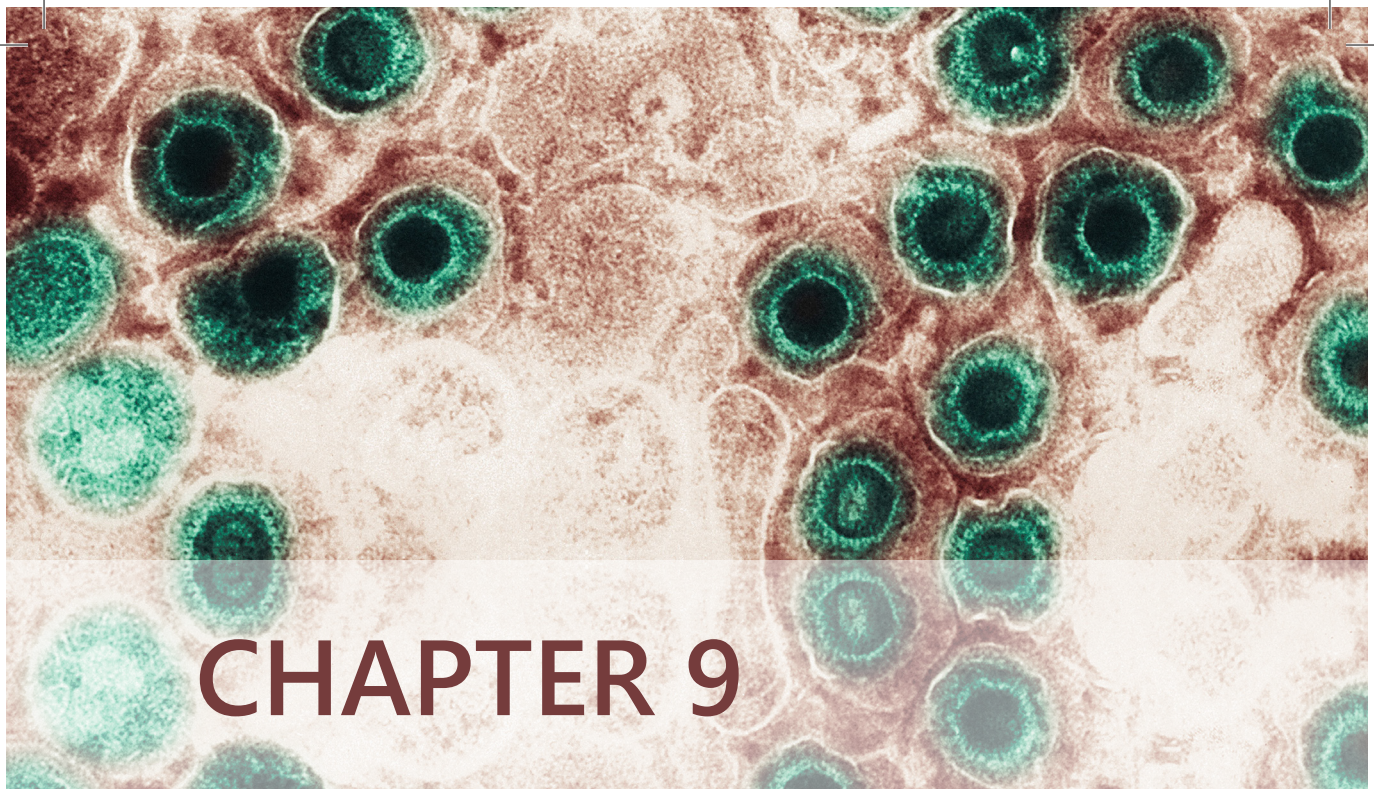
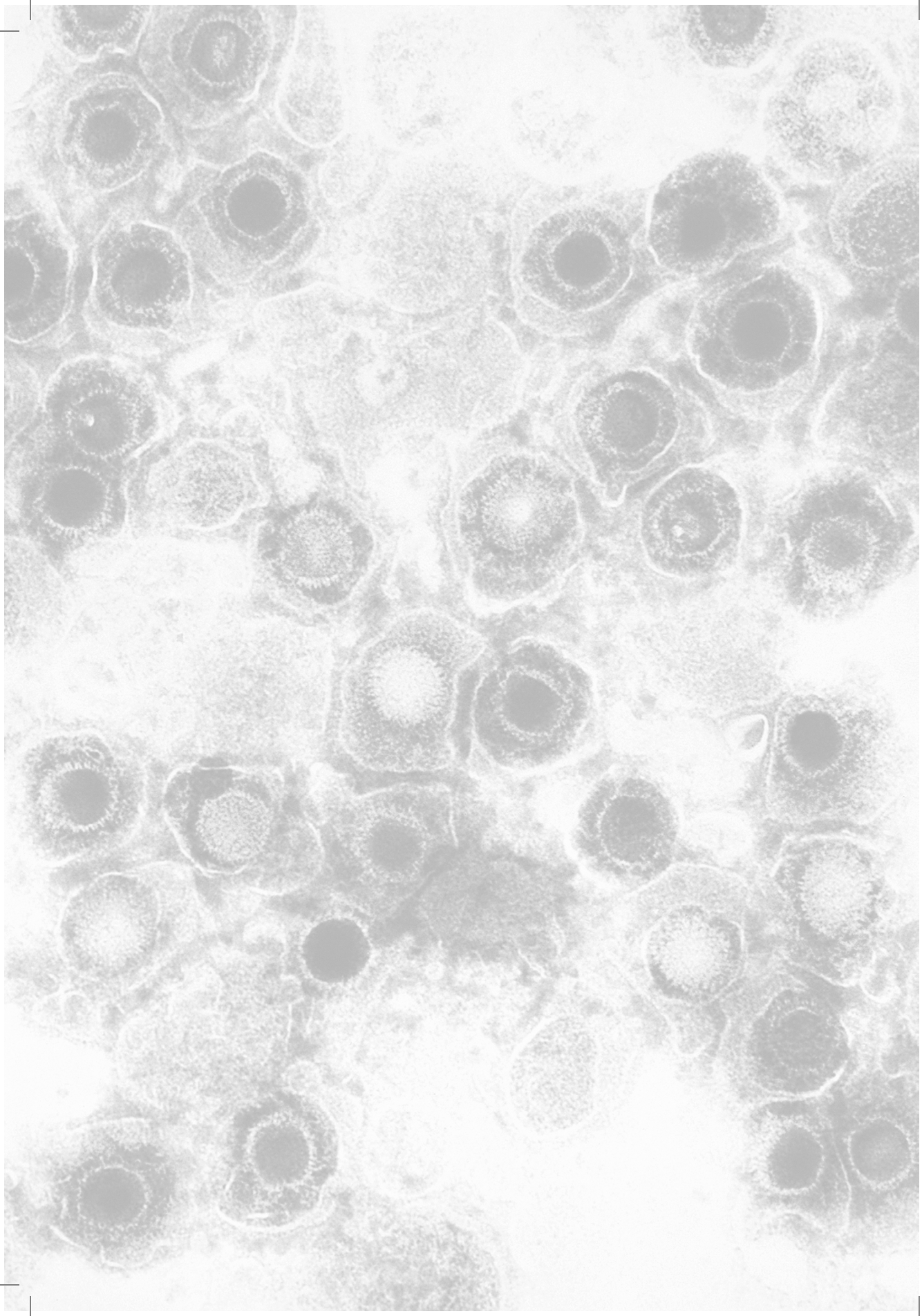


CHAPTER 9

Summary and general discussion
Recommendations for future research



CHAPTER 9

Summary and general discussion
Recommendations for future research

Summary and discussion

Cytomegalovirus infection is one of the most common fetal and neonatal viral infections.[1] In recent years, considerable advances have been made in our understanding of the epidemiology, pathophysiology and long-term burden of disease of both congenital and postnatal CMV infections. Congenital CMV (cCMV) infection is a prevalent viral infection in the neonatal population, with an estimated global birth prevalence of 0.7%.[2] It is a common cause of cerebral palsy and the leading cause of non-genetic sensorineural hearing loss (SNHL) and may cause significant long-term impairment in both symptomatic and asymptomatic children.[2–4] Early recognition of the infection may benefit neurodevelopment of the infant through immediate therapeutic interventions and regular follow-up. However, general awareness of this infection appears to be low.[5–7] National registries with the aim of prospectively studying disease burden have reported low registration rates.[8,9] In the Netherlands, several screening studies have been conducted to estimate the incidence/prevalence,[10,11] but no cCMV registry has so far been implemented.

Postnatal CMV (pCMV) infections occur frequently amongst neonates with an estimated median incidence of 20% and are generally uneventful in full-term neonates.[12]. Although the majority is asymptomatic, preterm infants and/or very low birthweight (VLBW) infants may develop symptomatic CMV disease.[13,14] Recently, several studies have published data on short- and long term outcome of preterm infants with pCMV infection, with inconclusive results.[13,15,16]

The aim of Part I of this thesis was to gain insight into the pitfalls of antenatal diagnosis of cCMV, and assess the current standards of infant recognition and awareness of cCMV infections in clinical practice. Furthermore, we wanted to determine the disease burden and evaluate postnatal neuro-imaging findings in correlation to the time of maternal infection during gestation and subsequent short-term outcome. In Part II, we focused on preterm infants with pCMV infection with the aim of evaluating available diagnostic methods for rapid diagnosis, describing neuro-imaging findings and to determine long-term neurodevelopmental outcome.

Part I — Congenital CMV infection

Diagnosing CMV infection after detection of fetal ultrasound abnormalities

In **Chapter 2** we have reported five cases with fetal ultrasound abnormalities detected in the second and third trimester suggestive of cCMV infection. All cases had varying degrees of cerebral- and extra-cerebral signs as seen on routine ultrasound scans around 20 weeks of gestation. Incorrectly, vertical transmission was considered unlikely, as maternal serology at this time indicated CMV infection in the past in all cases with negative CMV-IgM. The diagnosis of cCMV infection was eventually made in all cases after birth due to clinical symptoms or fetal autopsy findings. Subsequently, maternal first trimester serum was tested retrospectively, revealing a classic primary infection with positive CMV-IgM in case one, an unknown type of infection

in case two as first trimester serum had already been discarded, a probable primary infection in case three and non-primary infections in cases four and five, highlighting the pitfalls of maternal CMV-serology interpretation and the necessity for multiple diagnostic steps.[17] We have summarized our management recommendations in Figure 1. These recommendations serve as a diagnostic guide to the clinician trying to elucidate the etiology of fetal ultrasound abnormalities when CMV is suspected. When antenatal suspicion of cCMV infection arises and/or the infant has CMV-associated symptoms after birth, the infants' urine should be tested ≤2-3 weeks after birth to confirm cCMV infection.

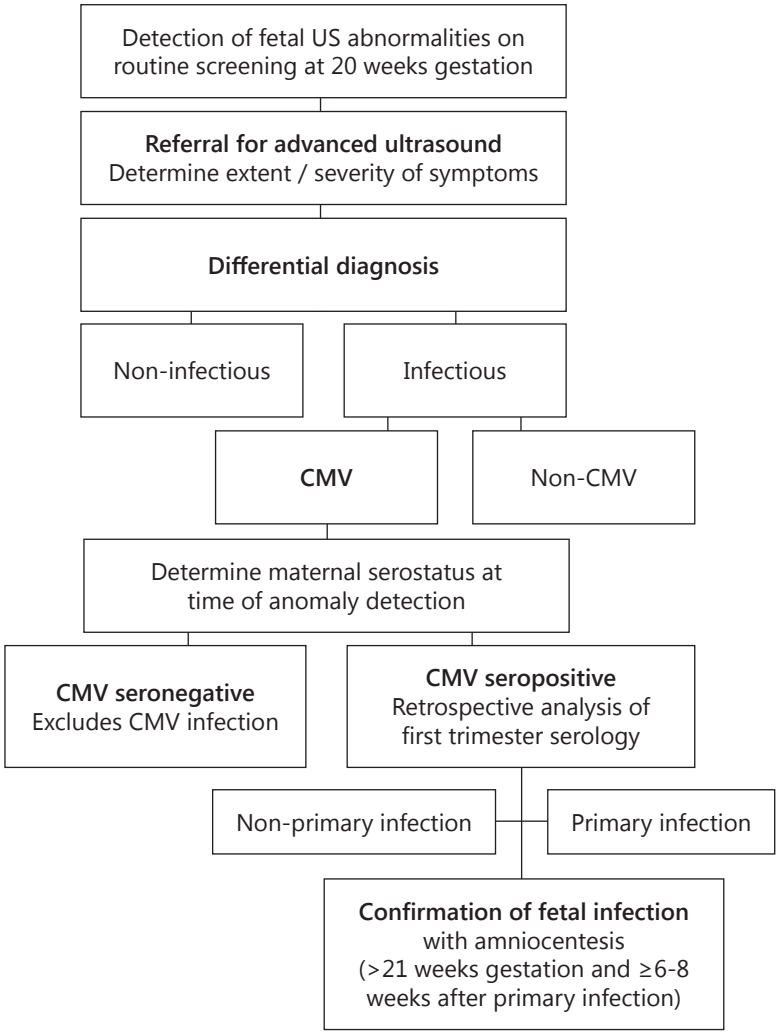


Figure 1. Management recommendation for diagnosis of CMV infection in the presence of fetal ultrasound abnormalities.

Congenital CMV infection in relation to trimester of maternal infection

In **Chapter 3**, we described the neuro-imaging findings and outcome of infants with cCMV infection in relation to the trimester of maternal infection and evaluated the additional value of MRI in depicting CMV-associated brain pathology. We have assessed the relationship between early maternal infection and the development of migrational abnormalities and neurological sequelae. The majority of infants born after a first trimester infection were symptomatic at birth and of these, most had severe abnormalities on cranial ultrasound (cUS) and MRI resulting in death, cerebral palsy, epilepsy and/or cognitive impairment. Infants without neuro-imaging abnormalities all had a normal outcome at 16-18 months of age, regardless of the presence of symptoms at birth. The significance of mild abnormalities remains to be determined. In this study, amongst the first trimester group with mild abnormalities, one (symptomatic) infant developed normally and one (asymptomatic) infant had bilateral SNHL and a mild cognitive delay. Infants born after second trimester infections had either no- or mild neuro-imaging abnormalities and all had a normal development except one symptomatic infant with bilateral SNHL requiring hearing aids. All children born after a third trimester infection had a normal outcome despite one infant with mild cUS and MRI abnormalities. We did not find MRI abnormalities in the absence of cUS abnormalities. Additionally, MRI was found superior to cUS with respect to the detection of migrational disorders, which where, when present, invariably associated with adverse neurodevelopmental outcome and/or development of SNHL. When only using cUS, failure to detect these severe lesions may occur and the severity of cerebral involvement may be underestimated. cUS was however superior at detecting LSV and germinolytic cysts demonstrating that both imaging modalities are complimentary.

Awareness and recognition of congenital CMV infection

In **Chapter 4** we aimed to determine the current practices surrounding recognition and management of infants with cCMV infection through an online survey amongst a select group of experts. Concomitantly, we assessed these aims through a national surveillance registry amongst Dutch pediatricians for cases of (symptomatic) cCMV infection.

Responses from the online survey came primarily from neonatologists with a special interest in neonatal neurology. Overall, there was lack of uniformity in the responses regarding screening, symptomatology, implementation of antiviral therapy, treatment duration and follow-up management. Maternal screening and infant screening were not standard care in any country and this was equally shown in the results of the questionnaire study (27% versus 5%, respectively). Remarkably, 65% of respondents did not consider CMV diagnostics when confronted with one or more CMV-associated symptoms. Furthermore, the hallmark symptom of cCMV infection, hearing loss, was not chosen by 16% of the respondents. Standard cranial MRI was performed in less than half of the responses and about one third performed MRI only when abnormalities were detected on cUS. In terms of antiviral treatment, the majority indicated treatment of symptomatic infants, with a small percentage also indicating treatment of asymptomatic infants. Lack of uniformity was also observed in treatment duration,

with the majority of the respondents treating for six weeks, however, treatment of 12 months was also noted. Furthermore, a considerable amount of respondents indicated that they did not know the duration or gave no answer. Audiological follow-up was carried out in almost all symptomatic infants, however, not in all asymptomatic infants and the timing of the follow-up also varied. Again, a considerable amount of respondents also indicated not knowing the frequencies of follow-up or gave no answer. Neurodevelopmental follow-up of all infected infants was standard procedure amongst the majority of the respondents; however some indicated no follow-up or did not give a response.

The national surveillance registry amongst Dutch pediatricians was conducted over three years. With approximately 515,000 infants born in the Netherlands over this period and a national birth prevalence of approximately 0.5%, of which 13% will be symptomatic, we would have expected roughly 330 children to be registered (Figure 2). Over the time span of the registry, only 55 registrations were made of which 48 could be included in the analysis. The majority of the registered infants were symptomatic with CNS-involvement, with the inclusion of five asymptomatic infants that tested positive after birth as a consequence of antenatal suspicion for CMV. Similar to the survey study, all treated infants were symptomatic infants and treatment duration also varied. Considering the estimated prevalence of cCMV infection in the Netherlands (0.5%[11]), we have shown that around 80-90% of the expected symptomatic infants with cCMV infection were not registered and that practices in management varied between hospitals in The Netherlands.

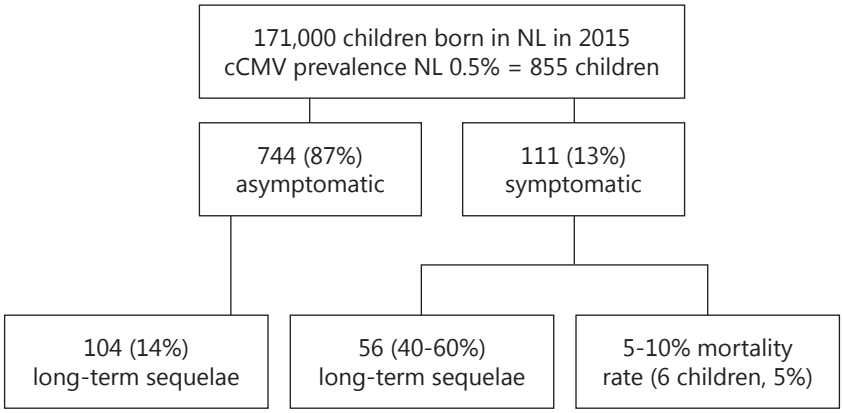


Figure 2. Total number of infected infants with cCMV infection in the Netherlands (based on the total number of live births in 2015).

Discussion — Part I

In Part I of this thesis, we have confirmed that despite considerable advances, there is lack of awareness and consensus on management of cCMV infection amongst health care professionals (**Chapter 2** and **Chapter 4**). This is supported by our findings in **Chapter 2** whereby all fetuses with anomalies suggestive of cCMV infection had delayed diagnoses due to incorrect interpretation of maternal serology results prior to birth. These cases serve as a reminder of the varying presentations of cCMV infection and the difficulties in proper and timely identification.

Neuro-imaging - management and outcome

CMV is a neurotropic virus and has the potential to infect almost all cell types of the brain, demonstrated by the high rate of CNS-abnormalities in symptomatic infants.[18] The exact neuropathogenesis of cCMV infections and the mechanisms leading to different phenotypic expression of CNS-sequelae however, remain poorly understood. Normal cortical development is the balanced result of proliferation of neural progenitor cells, neurogenesis and neuronal migration[19] occurring at different stages throughout the first half of pregnancy. Transmission of CMV during this time may interfere with brain development and consequently, lead to the development of migrational disorders. Congenital CMV infections, following first trimester primary infection have the highest incidence of SNHL[20] and fetal cerebral abnormalities, which is a strong predictor for neurodevelopmental outcome.[21,22]

It is therefore, essential to carefully examine the brain after birth in both symptomatic and asymptomatic infants. cUS is an ideal, non-invasive tool to make an initial assessment[23,24] and as we have shown in **Chapter 4**, is largely part of standard care in infected infants. In **Chapter 3**, we have additionally confirmed the value of cerebral MRI in depicting migrational disorders not detected by cUS and confirmed the association of severe cerebral lesions on both cUS and MRI to first trimester maternal infections. The impact of non-primary infections in the development of severe fetal anomalies as seen in **Chapter 2**, in relation to trimester of infection remains to be determined.

MRI was not carried out as part of standard care as frequently as cUS (**Chapter 4**), however is highly recommended even when cUS only shows mild-severe abnormalities (**Chapter 3**). Previously, cranial CT scans have been regularly used to identify calcifications.[25] We have shown that CT scans are no longer standard care (**Chapter 4**) and we recommend the exclusive use of cUS and MRI, as the first allows diagnosis of calcification and germinolytic cysts and the latter provides more detailed information about migrational disorders and does not use radiation (**Chapter 3**).[23,24]

Awareness and management

Despite the relatively small and select group of experts surveyed in **Chapter 4**, the results suggested that (symptomatic) infants may be under-recognized, especially considering the low registration rate in the national surveillance study. Although the results of the survey may not be generalized, it does offer some insight into the care of infants with cCMV infection. It was rather concerning to note the lack of uniformity in the responses amongst the experts especially regarding the choice of cCMV symptoms that would prompt diagnostics. Symptoms of cCMV infection are frequently mild and aspecific,[26] however, 16% of the respondents did not choose SNHL, a key disease characteristics. This suggests a lack of awareness and perhaps knowledge. Together, this underscores the difficulty of recognizing cCMV infection, even amongst experts and that may warrant the implementation of universal screening of infants.

Amongst the responses on management practices, varying approaches were also observed. Neuro-imaging in infants with cCMV infection is paramount in detecting potential cerebral lesions and counseling parents on infant outcome (**Chapter 3**) and the majority of the respondents also found neuro-imaging important. This however, could have been biased by the fact that most respondents had an interest in neonatal neurology. In terms of treatment with (val)ganciclovir, it was noted that some respondents also treated asymptomatic infants despite lack of efficacy data in this population.[27,28] Duration of treatment varied between 6 weeks and 12 months. There are no studies that have examined a 12 month course of antiviral treatment, again highlighting the lack of uniformity in management practices of the same disease. Differences in treatment duration were also found in the national surveillance registry despite the presence of a national CMV protocol in the Netherlands.[29] Incorporating more information on cCMV infection into obstetric and pediatric residency- and continuing education programs may provide more awareness and recognition of this disease amongst future generations of health care providers.

cCMV registries

Previously, several registries have been conducted to study disease characteristic of cCMV infections but similarly to our registry, all have consistently reported low registration rates (Table 1).

We think that this low registration rate stems from a combination of missed-diagnoses and under-reporting. The success of registries is mainly reliant on the compliance of doctors to report infants. Under-reporting may factor into the low registrations rate due to lengthy questionnaires in the setting of an already busy clinical practice. Although the spectrum of clinical symptoms that may arise in cCMV infection has been well documented, it seems through the low registration rate in our national registry that recognition may not be as straightforward. Recently, in a retrospective nation-wide study in the Netherlands, merely 2.6% of all infected children had the diagnosis of cCMV infection at 5 years of age, despite clinically apparent symptoms in the neonatal period in 18.1%.[11]

Table 1. Congenital CMV registries around the world.

Country	Results	Study conclusions and limitations
USA 1990 – 1993	Prospective voluntary reporting 285 symptomatic registrations; 268 confirmed, 17 possible cases. 1.7% infants registered of estimated total.[8]	• voluntary participation • passive reporting
Japan 1992 – 1993	National survey 49 confirmed cases. Estimated incidence: 3.9/100,000 Study incidence: 1.6/100,000.[127]	• missed diagnosis • due to high seroprevalence less symptomatic cases
Australia 1999 – 2002	National pediatric surveillance unit 153 registrations; 62/1500 (4%) confirmed cases. Study estimate: 1500 cases annually.[30]	• missed diagnosis • limited number of reporting doctors
UK & Ireland 2001 – 2003	National pediatric surveillance unit 290 registrations, 86 confirmed cases, 70 possible cases. Estimated prevalence: 3/1000 Study prevalence: 0.06/1000[31]	• non-specific clinical presentation, missed diagnosis • delay in diagnostics
Canada 2005 – 2008	National pediatric surveillance unit 118 registrations; 49 confirmed cases. Estimated 80% not registered.[9]	• missed diagnosis • under-reporting
Belgium 2007 – ongoing	Flemish CMV registry 6 Flemish centers. www.cmvreg.be	

In a review of medical records for cases of cCMV infection in British Columbia, Canada over a time span of around 8.5 years, around >90% of symptomatic cases were estimated to have been missed.[32] It has been suggested that the severity of symptomatic cCMV infection may be overestimated due to selection bias when comparing screened infants with referred infants.[26] The relevance and scope of mild-moderate symptoms let alone the impact of asymptomatic infections can only be gathered from large scale newborn screening studies.

The Centers for Disease Control (CDC) defines public health surveillance as the "ongoing, systematic collection, analysis, interpretation, and dissemination of data regarding a health-related event for use in public health action to reduce morbidity and mortality and to improve health".[33] Based on our findings and on previous registries, we believe the aims of studying cCMV infection through these means are currently not achievable.

Targeted screening versus universal screening

CMV has been recognized as a public health issue of major concern[34] and recently the benefits of early CMV detection in cCMV infected infants has gained more momentum.[26,35–40] The hallmark of cCMV infection, SNHL, warrants close and repeated monitoring as its evolution is fairly unpredictable.[36,41,42] Recently, Korndewal et al. demonstrated that children with cCMV infection were twice as likely to develop long-term impairment up to six years of age.[3] Despite the lack of a curative therapy ((val-) ganciclovir) or means to completely prevent infection (vaccination, administration of CMV-hyperimmune globulins), early detection of cCMV infection has substantial benefits in terms of reducing the morbidity and lifetime costs of therapies and loss of productivity.[43–46]

Early detection of CMV after birth could be achievable through two means: targeted screening of infants who fail the Newborn Hearing Screening (NHS) or universal screening of all newborns at birth.

It has been shown that targeted screening can be carried out in a time frame that allows pediatric assessment and would still enable initiation of therapy.[28,38,47,48] The crucial shortcomings of this method however, have been recently demonstrated by Fowler et al. whereby despite identifying the majority of infants with CMV related SNHL, 43% were not identified by NHS.[38] Especially amongst the asymptomatic infants, NHS failed to identify 53% of the infants with SNHL. This is concerning, as around 21% of asymptomatic infants may develop some form of hearing loss.[36] Similar results were found in the first mandate targeted CMV screening program for infants failing the NHS in the state of Utah, USA whereby merely 14 infected infants were identified in two years.[48,49] Targeted CMV screening would be helpful in determining the etiology of hearing loss in infants with an unknown cause. In terms of identifying all infants with cCMV infection at risk for hearing loss and eligible for audiological follow-up, it does not suffice.

Universal screening for CMV at birth would detect all infants at risk for sequelae. Most importantly however, universal screening would address the issues of under-reporting and/or missed-diagnoses in registries (**Chapter 4**) and will allow for detection of asymptomatic infants.

In the event of neurodevelopmental sequelae, prior knowledge of CMV status may aid in determining the cause. This is important as these manifestations often coincide with an age when retrospective testing for CMV may not be possible anymore[4,50] or in countries where DBS cards are not stored for longer periods. In a review by de Vries et al. it was demonstrated that the Wilson and Jungner criteria for screening are largely met concerning screening for cCMV infection.[35]

Nevertheless, universal screening also has some disadvantages as it remains difficult to predict which infants will and will not develop sequelae. The ethical burden on families due to the uncertainty of the prognosis of their child is questionable, as

the majority of infants will remain truly asymptomatic. The impact of screening in causing parental anxiety must be weighed against the benefit of early diagnosis. The best approach would be to study the feasibility of universal screening in a large scale newborn screening study, coupled to a systematic, longitudinal follow-up program. This will allow for a prospective collection of data to provide more accurate population-based estimates of disease burden, allow for characterization of symptomatic and asymptomatic infants and potentially identify concrete risk factors for adverse outcome. Additionally, the psychological impact of universal screening should and can be assessed, with the main focus on parent-child bonding amongst asymptomatic children without long-term sequelae.

Means to screen

A frequent point of discussion in universal screening of infants for cCMV infection is what type of material is most suited for the purpose and how to implement a fast, high-throughput and reliable screening strategy. In spite of the fast and accurate analytical ability of CMV-PCR, several problems persist concerning appropriate choice of specimen (differences in viral loads) and the optimal collection, transportation and storage of specimens.

Urine

Isolation of CMV from urine or saliva culture is considered the gold standard for the diagnosis of cCMV infection.[51,52] This method is timely and requires specialized technical expertise, as such it has been increasingly replaced with CMV-PCR of urine.[53] Using urine for universal screening would be ideal, as it contains high viral loads and CMV-PCR is highly sensitive and specific.[53] The major disadvantage of urine however is the collection in sterile bags at risk for contamination.[54,55] An alternative to liquid urine would be urine collected on filter paper placed in an infant's diaper. This method has shown equal diagnostic agreement to testing of liquid urine and can be implemented in a high-throughput capacity.[54–56]

Saliva

Similar to urine, screening of saliva by means of CMV-PCR has shown to have excellent sensitivity and specificity when screening a large cohort of unselected infants.[57,58] Saliva samples are easy to collect and non-invasive by means of saliva swabs[57,58] or on saliva filter paper[56] from the infant's oral cavity. A disadvantage with this collection method is the necessity for planning the sampling long enough after the infant's feeding for risk of CMV contamination through breastmilk. This may however, be negligible if performed <1 week after birth.[59] Screening of saliva would require new infrastructure for collection and transport, especially with liquid saliva, which has a marginally higher sensitivity whereas dried saliva specimens would simplify this procedure.[37,57] Both methods could be implemented for high-throughput testing.[57]

Dried blood spots (DBS)
From a logistics standpoint, universal CMV screening would ideally be in-cooperated into the preexisting screening program by means of CMV-PCR on DBS cards. This would eliminate the need for new collection, transport and testing infrastructure. The specificity of this method has been reported to lie between 99.3%-100%.[60–62] The major concern with this method however is the wide range in testing sensitivity reported in the literature (34-100%).[50,60,61,63–65] This wide range has been attributed to differences in study population (selected versus unselected infants) and differences in PCR assay protocols, as well as size/input volume of tested DBS and DNA extraction methods.[37,65–68] Recently, Ross et al. demonstrated a low sensitivity and specificity of DBS CMV-PCR in detecting infants at risk for late onset SNHL.[50] Important to realize is that the results of this study are based on the PCR protocol by Boppana et al. which uses the M48 MagAttract Mini DNA extraction method,[63] which has been shown to yield low CMV-DNA loads when compared to other assays.[68] With the protocol by Barbi et al. the utility of DBS CMV-PCR has been demonstrated in a large unselected cohort of 509 children whereby a sensitivity of 100% and specificity of 99% were achieved when compared to viral culture of urine and/or saliva.[61] Similarly, Kharrazi et al. screened 3972 unselected newborn DBS cards and demonstrated a similar prevalence of cCMV infection as reported in the literature,[69] indicating that this method may be an effective means to screen.

A matter that merits further research is the impact of viral load on DBS sensitivity. Using a modified protocol by Barbi et al.[61] de Vries et al. did demonstrate a method with potential for high-throughput testing, reaching a sensitivity of 100% for specimens containing CMV loads of 5-4 $\log_{10}$ copies/ml, however this decreased substantially when testing specimens with lower viral loads.[66] The clinical relevance of these findings remains to be determined, as studies have shown that infants with lower viral loads have less chance of developing sequelae.[70–72] It remains to be seen if it is acceptable that infants with low viral loads are not detected, depending on the potential development of sequelae long-term sequelae in this group.

Follow-up of screened infants
A frequent concern of universal screening however, is what to do with the large volume of infants/children that are entering the medical circuit for follow-up. We have proposed a management scheme in Figure 3, for a possible pilot screening study for the Netherlands. In the Netherlands, all children are regularly seen at a child health care center (NL: *consultatiebureau*) to evaluate their development. In fact, on average children are seen for a 20-minute consultation around 12 times from the age of one month of age until nine years (https://www.ggdru.nl/mijn-kind/contactmomenten.html).

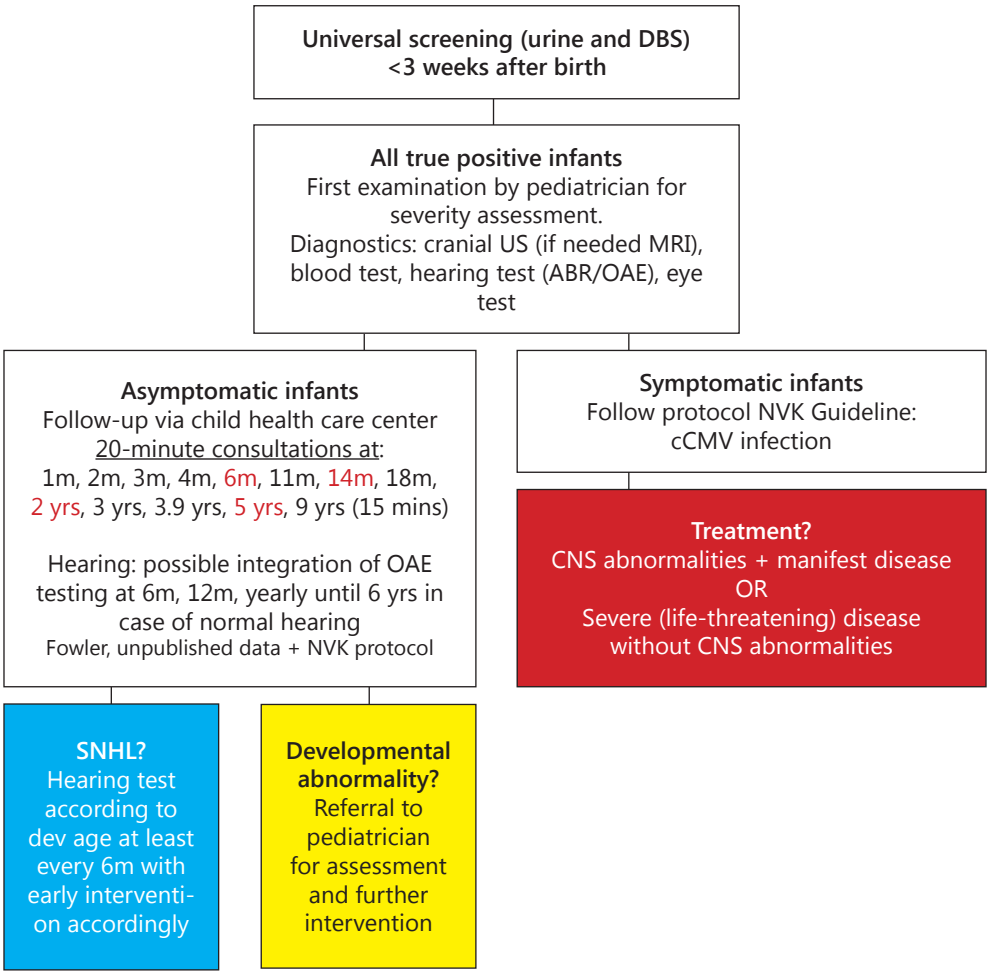


Figure 3. Screening management scheme for the Netherlands. Abbreviations: OAE: otoacoustic emission; ABR: auditory brainstem response; SNHL: sensorineural hearing loss; CNS: central nervous system

Screening of infants may be performed by urine and DBS CMV-PCR within the first week after birth. By using urine (gold standard) the risk of false positive or negative results will be eliminated. All true positives would be referred for an initial examination and severity assessment by a pediatrician. This exam would include a cUS and if needed a cranial MRI scan, blood tests, hearing test (if not already tested by the newborn hearing screening) and ophthalmological examination (eye examinations in both asymptomatic and symptomatic infants for the duration of the pilot screening study). Symptomatic infants that meet the criteria may be treated with (val)ganciclovir and followed-up according to the national cCMV protocol.[29] Special

treatment protocols could be set up to assess the necessity of treating mild-moderate symptomatic cCMV infection. Infants that do not have any overt signs of cCMV infection, that are truly asymptomatic could be followed-up through the child health care center at time intervals of every six months in the first year and then annually until ±6 years of age. As proposed by Fowler et al. at the CMV Public Health & Policy Conference in Austin, TX, USA in September 2016, asymptomatic infants could have their hearing tested by otoacoustic emission testing (OAE) (Figure 2), which could be completed during a health checkup at the child health care center. By screening all infants and allowing for a pediatric check-up and risk assessment, parental anxiety may even be reduced as initial risk factors such as cerebral abnormalities would be immediately assessed. In any case, the impact of screening on parental/ child stress and child bonding should be equally evaluated in the benefit assessment of universal screening for cCMV infection.

Part II — Postnatal CMV infection

Risk factors for transmission and predictors of severity

In **Chapter 5** we reviewed the literature to summarize the current knowledge of the epidemiology, transmission and clinical presentation of pCMV infection in preterm infants, identify risk factors of transmission and predictors of clinical severity and discuss the application of preventive and therapeutic measures.

Epidemiology and transmission

A pCMV infection in preterm infants (<32 weeks GA) is estimated to occur with a incidence of approximately 20%.[12] Transmission of CMV can occur through contact with any infected bodily fluids but most frequently occurs due to shedding of CMV in breastmilk of CMV seropositive mothers.[73] The risk factors associated with transmission can be broadly divided into viral (transmission route) factors and host factors. Of the seropositive mothers that will transmit the virus to the infant, around 96% will have CMV-DNA or full virions in their breastmilk.[73] Not all mothers that shed CMV in breastmilk will transmit the virus to their infants (transmission rate up to 76%).[14,73,74] Although the pathophysiology remains unclear, transmitting mothers tend to shed CMV in breastmilk at an early stage after birth[73,75] and have a higher viral load compared to non-transmitters.[76] Furthermore, a low anti-CMV IgG infant-mother ratio (a measure of maternal-to-infant antibody transfer) correlates to a lower GA and is associated with a higher risk of transmission.[77]

Severity

Gestational age (GA) and birthweight are important predictors of clinical severity of pCMV infection. Extremely preterm infants (GA<28 weeks) are more prone to develop more severe symptoms such as CMV-sepsis-like-syndrome (CMV-SLS), whereas preterm infants with a higher GA/BW tend to be asymptomatic or have transient laboratory abnormalities.[78,79] It has been observed that pCMV infection may cause

more symptoms in infants with pre-existing comorbidities, such as extremely preterm infants.[79,80] As such, CMV has been implicated as a co-aggravator of an already vulnerable patient whereby severe symptoms are secondary to preterm morbidities. Currently, there are no associations between host viral load and/or CMV genotypes as predictors of severity.

Treatment and prevention
There are currently no controlled studies investigating the efficacy and safety for the use of (val)ganciclovir in (extremely) preterm infants with pCMV infection. Incidental use of ganciclovir has been described in infants with severe, life-threatening pCMV infection in the absence of other treatment options.[79,81] Prevention of transmission in infants at risk may be achieved through withholding or pre-treating breastmilk by means of freeze-thawing[82] or different methods of pasteurization.[83–85]

Screening for pCMV infection using urine and saliva

After CMV infection, shedding of CMV can be observed in various bodily fluids, amongst which urine, saliva and blood.[86,87] The gold standard for diagnosing CMV infection is considered virus isolation by cell culture from urine or saliva.[51,52] More recently however, CMV-PCR of urine is increasingly used due to its excellent sensitivity.[53] Also, screening saliva for CMV-DNA by means of PCR has shown high sensitivity and specificity amongst term infants with cCMV infection.[57] In Chapter 6 we compared screening of saliva to urine,[53] by means of quantitative real-time PCR in detecting pCMV infection amongst preterm infants.

In total 261 paired saliva-urine samples, collected at term-equivalent age (TEA), were analyzed. The vast majority of the infants received fresh breastmilk from their mothers. A pCMV infection was detected at TEA by rtPCR of urine and saliva in 18% and 16.1% of the infants, respectively. Saliva rtPCR failed to identify five (10.6%) asymptomatic infants with pCMV infection. Furthermore, a false positive result was observed with saliva rtPCR in one (0.5%) infant, which may be caused by contamination of saliva with CMV infected breast milk. The five infants with a false negative result had a significantly lower median viral load in urine compared to infants who had a positive result on both assays. Nine urine rtPCR positive (19.1%) infants who were symptomatic were all also positive on saliva rtPCR. On the basis of these data we determined that the general sensitivity of saliva rtPCR was 89.4% and the specificity 99.5%. When including only symptomatic infants however, the sensitivity increased to 100% with a specificity of 99.5%. Conversely, when including only asymptomatic infants in the analysis the sensitivity dropped to 86.8% and the specificity remained the same at 99.4%.

White matter microstructure in preterm infants with pCMV infection

It has previously been demonstrated that the presence of lenticulostriate vasculopathy (LSV) at TEA was significantly more often present in preterm infants with pCMV infection than non-infected controls, suggesting CNS involvement.[88] In Chapter 7 we examined the integrity of the white matter in both hemispheres of the brain within

three regions of interest by means of diffusion tensor imaging (DTI) at TEA between CMV positive (n=21) and CMV negative (n=61) preterm infants (median GA 26.3 weeks versus 27.4 weeks, respectively). For each of these regions of interest, axial-, radial- and mean diffusivity were calculated, as well as fractional anisotropy, which are measures of water diffusion and assess microstructure of the white matter of the brain.

We demonstrated that the median axial-, radial- and mean diffusivity scores were generally increased and median fractional anisotropy was decreased amongst preterm infants with pCMV infection compared to non-infected infants. This difference only reached significance in the occipital region. To determine the significance of these findings, we compared the outcome of non-infected infants (60/61; 98%) and infected infants (19/21; 90%) at 16 months corrected age (CA) using the Griffiths Mental Development Scales (GDMS) and found no significant differences in outcome between both groups.

Long-term outcome of preterm infants with pCMV infection

Short-term outcome among preterm infants with a pCMV infection appears to be unaffected.[89,90] More recently however, a negative impact on long-term outcome has been suggested.[91–93] To elucidate the short- and long term sequelae of a pCMV infection in the preterm population, we prospectively compared the neurodevelopment between infected and non-infected infants until 6 years of age in **Chapter 8**. Neurodevelopment was assessed at three points in time, corresponding to the age of 16 months CA, 24-30 months CA and 5-6 years of age. Additionally, we examined hearing amongst preterm infants with a pCMV infection at 6 years of age. Amongst the pCMV infected infants, four manifested symptoms in the neonatal period, and one subsequently died at six months due to complications not associated with the pCMV infection.

At 16 months CA a total of 356 infants were assessed of which 49 were infected and 307 were non-infected infants. No significant differences were noted on any subscales of the GMDS except the locomotor subscale, whereby infected infants scored higher than controls. The mean general developmental quotient was comparable between both groups. At 24-30 months CA no differences were noted on any subscales using the GMDS or the Bayley Scales of Infant and Toddler Development-III (BSITD-III). Interestingly, we noted that infants with a pCMV infection actually started walking independently significantly earlier than non-infected infants. Multivariable regression analyses showed that this observation was attributable to maternal non-Western ethnicity. At 6 years of age, infected children had mean scores in the normal range but generally scored lower, on all subscales of the Wechsler Preschool and Primary Scale of Intelligence-III (WPPSI-III) than non-infected children. The only statistically significant difference was noted on the verbal subscale, which was significantly impacted by non-western maternal origin and low maternal education. Postnatal CMV infection did not significantly contribute to any of the variance observed. Sub-analysis of symptomatic versus asymptomatic pCMV infection showed no sig-

nificant differences with respect to neurodevelopmental outcome. Motor function at 6 years of age was assessed using the Movement Assessment Battery for Children-II (MABC-II) and was comparable in both groups. Lastly, hearing examinations until 6 years of age indicated no signs of SNHL in infected children. Audiological outcome of this cohort at 1-2 years of age has been previously described,[94] and equally did not indicate SNHL.

Discussion — Part II

Neurodevelopmental outcome of preterm infants with pCMV infection remains a subject of discussion due to conflicting results of previous studies and lack of management guidelines. Focus is especially placed on the preterm population as (extremely) preterm infants may be comparable to third trimester fetuses with cCMV infection. Preterm infants and/or very low birthweight infants have been shown to be at increased risk for symptomatic pCMV infection as reviewed in **Chapter 5**. Despite these risk factors, a central question regarding the management of pCMV infected infants is, what the precise impact of these symptoms is on the short- and long-term development and if preventive and/or therapeutic measures are warranted in order to prevent or even treat pCMV infection.

We extensively and prospectively studied the largest cohort of preterm infants from birth until 6 years of age for potential effects associated with pCMV infection. All included infants received fresh and untreated breastmilk from their mothers, which can be assumed to be the main route of transmission due to the sole use of CMV seronegative blood products at our NICU.[95]

Neuro-imaging and outcome

In cCMV infection the most crucial predictor for adverse neurodevelopment is the presence of intracranial abnormalities as we have confirmed in **Chapter 3**. The development of brain lesions is closely coupled to the intricate timing of proliferation and differentiation of neuronal cells.[18,96,97] Mainly during the third trimester, pre-myelinating oligodendrocytes are vulnerable to inflictions such as infection and inflammation resulting in white matter injury due to disturbed myelination.[96] The presence of white matter abnormalities at TEA in preterm infants has been associated to a poorer neurodevelopmental outcome.[98,99]

We have examined the neuro-imaging findings in our cohort study of pCMV infected infants by means of cUS, cranial MRI and DTI. Previously, the findings of cUS at TEA and cranial MRI at TEA revealed no gross lesions in the brain.[88,95] On cUS, however infants with pCMV infection had significantly more LSV than non-infected infants.[95] The presence of LSV, however is not pathognomonic for CMV and is frequently found amongst preterm infants due to a broad range of etiologies.[100] The clinical/prognostic significance of LSV remains largely unclear.[101,102] In a recent study from Israel,

Bilavsky et al. suggested that LSV is a high-risk marker for hearing loss in infants with cCMV infection that warrants antiviral treatment.[103] In our study, we demonstrated that despite the significant presence of LSV at TEA amongst preterm infants with pCMV infection, no adverse effects on neurodevelopment until 6 years of age were observed (**Chapter 8**). None of the infants were treated and a sub-analysis between infants with and without LSV at TEA equally showed no difference in neurodevelopmental outcome, including hearing.

To further examine the integrity of the white matter we analyzed DTI scans of preterm infants with pCMV infection. DTI is increasingly applied to assess the white matter and appears to be correlated to neurodevelopmental outcome of neonates.[104] We only found signs of impaired white matter microstructure in the occipital region of interest in the brain (**Chapter 7**). The significance of this and the pathophysiological mechanism remain elusive. Cerebral visual impairment has not been described in pCMV infection. Pre-myelinating oligodendrocytes may be directly affected by (CMV) infection or through injury to other cells or disturbances in cerebral development leading to impaired white matter myelination.[96] These disturbances may cause low FA values, which reflect damage or immaturity of the white matter.[104] The direct causal relationship between pCMV infection and focal occipital white matter injury however, is difficult to discern. Gross damage to the occipital white matter region may result in abnormal visual acuity and impaired development of visual fields, but only mild changes in white matter microstructure were identified in our patients.[105,106] Although infected children did not have visual function assessment at 6 years of age, they had a higher median score on the subscale of ball skills of the MABC-II at 6 years of age, compared to non-infected controls (**Chapter 8**). The tasks of this subscale require the child to throw and catch items accurately, requiring intact visual acuity. We were therefore, unable to show any adverse effects of the occipital white matter abnormalities seen at TEA on outcome at 6 years of age.

Consequences and outcome of pCMV infection amongst preterm infants

Although pCMV infection usually remains asymptomatic, risk factors such as (extremely) preterm birth, VLBW and early transmission may forecast the development of symptoms in the neonatal period (**Chapter 5**). We observed amongst infants with pCMV infection (n=74) in the neonatal period, that only four infants (5%) developed symptoms. This rate of symptoms is in accordance with reports in the literature (median 3.7%; range: 0 – 34.5%).[12] It is important to note that all symptoms were mild, transient and self-limiting and did not require antiviral treatment.

In a recent retrospective study from Germany, Mehler et al. observed a high rate (48%) of symptomatic pCMV infection in infants born with a GA between 22-24 weeks.[79] Among 11 symptomatic infants, four (36%) had transient and self-limiting thrombocytopenia as the only symptom. Six infants developed CMV-SLS with additional symptoms, of which three were treated with (val)ganciclovir. One infant had isolated thrombocytopenia but was treated with (val)ganciclovir due to severe immaturity (GA 22 weeks). All infants eventually recovered, and milder symptoms were self-li-

miting, as we have also found in our study (**Chapter 8**). In the Netherlands, active perinatal support is recommended after a GA of 24 weeks,[107] and we therefore did not have any infants born with a GA<24 weeks at our NICU. In countries that provide active neonatal intensive care to infants born with a GA 22-24 weeks, the long-term clinical impact of symptoms as a result of pCMV infection remains to be determined. Based on our results, pCMV did not affect cognitive- or motor development until 6 years of age. There was no difference between extremely preterm (GA>24 weeks) infants with pCMV infection who were symptomatic or asymptomatic or between infected and non-infected children.

The incidence of pCMV infection amongst our cohort of 462 eligible infants was 16%, a rate higher than a recent prospective multicenter study from the USA, whereby 539 VLBW infants were screened and 5.4% had pCMV infection.[108] Unlike our cohort where all infants received untreated breastmilk, the majority of the infants from the study by Josephson et al. exclusively received freeze-thawed breastmilk, which would explain the lower rate. Amongst the infected infants, 17.2% developed symptoms, a rate much higher than we observed. Of the symptomatic infants (n=5), three developed necrotizing enterocolitis (NEC) and died as a consequence. The development of NEC after pCMV infection has only been described occasionally in the literature (**Chapter 5**). It is difficult to distinguish between prematurity and pCMV infection as a cause of the NEC. Some suggest that due to CMV-induced modulation of the host immune response, disturbances in the immunologic barrier within the gastro-intestinal mucosa may occur and cause susceptibility to NEC.[109] On the other hand, it has been demonstrated that the occurrence of NEC is inversely related to the amount of untreated breastmilk a preterm infant receives.[110] In our large cohort study, we observed no occurrence of NEC or mortality amongst extremely preterm infected infants who all received untreated breastmilk. When the infants with NEC are removed from the study by Josephson et al., then 6.9% are symptomatic, similar to the rate of 5% in our cohort (**Chapter 8**).

As previously mentioned in this thesis, there are uncertainties regarding the impact on neurodevelopment of a pCMV infection amongst preterm infants. Previous studies on neurodevelopment report conflicting results and are frequently based on small sample sizes. To thoroughly investigate this matter, we prospectively screened a large cohort of preterm infants and examined neurodevelopment for the effects of a pCMV infection from birth until 6 years of age. Despite the presence of established severity factors (preterm GA <29 weeks, VLBW <1500 grams) (**Chapter 5**) in our cohort, we have demonstrated no severe CMV disease or negative consequences on neurodevelopment as a result of pCMV infection (**Chapter 8**).

In contrast to our findings, a German research group reported a negative impact on long-term cognition as a result of pCMV infection in preterm infants, that became especially evident in adolescence.[13,93] In general, it remains difficult to study neurodevelopment, considering the numerous amounts of confounders, methodological differences in assessing neurodevelopment and frequently encountered loss to fol-

low-up. Interestingly however, between the German cohort and our cohort is the trend that infected children seemed to have lower mean scores on intellectual tests. While they ascribed this to CMV status, we did not find this association amongst our larger cohort of children. Rather, we found that our observed variance was due to familial factors, factors that were not controlled for in the German study, like maternal ethnicity.[111] In conclusion, on the basis of the investigations in this thesis, pCMV infection did not lead to adverse effects in the neonatal period. Despite the development of LSV at TEA[95] and the presence of changes in the occipital white matter microstructure (**Chapter 7**), no consequences on neurodevelopment, including hearing were observed until 6 years of age (**Chapter 8**).

Management recommendations

We have shown in a large prospectively screened cohort of extremely preterm infants that the incidence of symptoms as a result of pCMV infection is low and that none of the infected infants required administration of (val)ganciclovir (**Chapter 8**). Incidental reports of a short-term course of (val)ganciclovir in severely symptomatic infants have been described, allowing for recovery of clinical parameters.[13,79,109,112] The efficacy and safety of (val)ganciclovir amongst preterm infants with pCMV infection has not been studied in a randomized controlled trial. In the meantime, pending results of such a study, administration of (val)ganciclovir should be determined on a case-by-case basis and reserved only for situations of severe clinical deterioration (**Chapter 5**).

We have shown that administration of untreated breastmilk and subsequent acquisition of pCMV infection did not have a negative impact on neurodevelopment (**Chapter 8**). Especially amongst preterm infants, the use of fresh breastmilk is strongly recommended as it provides imperative nutritional and immunological benefits for growth and development.[110,113–115] Pre-treatment of breastmilk in the general preterm population with the aim of minimizing the risk of CMV transmission is not justified by our results, although some exceptions may have to be made (e.g. extremely preterm infants, severe morbidities).

Recently, serial screening of preterm infants has been proposed as a preemptive strategy to detect pCMV infection and implement therapy before the development of symptoms.[116] As we have shown, the incidence of symptoms is low and symptoms that do arise are frequently mild and self-limiting (**Chapter 8**). In the absence of a clinical trial, routine administration of (val)ganciclovir seems extremely controversial. While almost all seropositive mothers shed CMV during the neonatal period, it would make more sense to preemptively screen the mothers for CMV seropositivity. Amongst seropositive mothers with extremely preterm infants (GA<24 weeks) or preterm infants with severe associated comorbidities, pre-treating breastmilk may be considered until a more clinically stable stage has been reached (**Chapter 5**). Pre-treatment by short-term pasteurization is most advisable due to complete CMV elimination and retention of breastmilk benefits.[82,84] Recently, a new and fast strategy of pre-treatment by means of microwave irradiation successfully eradicated CMV; however the impact on the nutritional value of breastmilk remains to be investigated.[117]

Conclusions

Based on this thesis, the following conclusions can be drawn:

1. Fetal abnormalities detected on routine sonography scans at 20 weeks gestation can also be the result of maternal non-primary infection. Delayed diagnosis can be avoided by increased awareness of CMV and correct diagnostic interpretation (**Chapter 2**).
2. Severe CMV-induced lesions of the brain are most frequently the results of first trimester primary infections and are predictive of adverse outcome amongst infants with a cCMV infection. The use of cUS and MRI are complimentary in the diagnostics of cCMV infection. Cranial US is superior to MRI in diagnosing LSV and germinolytic cysts, while MRI is superior to cUS in depicting migrational disorders (**Chapter 3**).
3. Assessment of recognition and management practices of infants with cCMV infection showed inconsistencies amongst a select group of experts. Key symptoms of cCMV infection, such as SNHL, are not recognized as a consequence of cCMV infection (**Chapter 4**).
4. The use of a national registry to document symptomatic infants with cCMV infection seems ineffective in studying disease burden and management practices (**Chapter 4**).
5. Risk factors for transmission of pCMV infection from mother to preterm infant are earlier shedding of CMV in breastmilk, a higher CMV load in breastmilk and a low anti-CMV IgG infant-mother ratio. Predictors that may forecast the development of symptoms in preterm infants with pCMV infection are extremely preterm birth (GA<28 weeks) and very low birth weight (<1500 grams) (**Chapter 5**).
6. When screening preterm infants for a pCMV infection, urine PCR is more reliable than saliva PCR, however for diagnostic purposes in infants with suspected pCMV infection (i.e. symptoms) saliva CMV-PCR is accurate (**Chapter 6**).
7. Preterm infants with a pCMV infection have reduced occipital FA values on DTI at TEA, suggestive of abnormal microstructure in the occipital white matter (**Chapter 7**).
8. Postnatal CMV infection among preterm infants is not associated with impaired long-term cognitive and motor neurodevelopment until 6 years of age (**Chapter 8**).
9. Hearing is not affected by a pCMV infection amongst preterm infants until 6 years of age (**Chapter 8**).

Future research

Universal screening of cCMV infections

In order to effectively recognize and study infants with a cCMV infection universal screening for CMV should be conducted. As previously proposed in this thesis, a universal screening program in the Netherlands should be feasible (Figure 3). Future efforts should be dedicated at conducting a pilot screening study. Screening of infants could be done by means of urine CMV-PCR (gold standard) and concomitantly by DBS CMV-PCR within the first week of life combined with the general newborn DBS screening. By using urine and DBS CMV-PCR, the analytical capacity of DBS screening could be optimally assessed and optimized in a large unselected group of newborn infants. Prior to this the DBS CMV-PCR protocol should be optimized using methods that have yielded high sensitivity and specificity. A cost-effectiveness assessment should be performed examining the financial costs/ advantages of the respective screening methods, as well as the benefit of early detection in respect to early implementation of antiviral- and assistive therapies (hearing aids, cochlear implants, speech-language therapy). Positive infants should be initially assessed by a pediatrician, after which follow-up of the large group of asymptomatic infants could be conducted through the routine developmental assessment at the child health-care centers that are already in place. Results from these initial assessments will reveal the full spectrum of symptomatic CMV disease and allow for a complete assessment of disease burden. Additionally, by analyzing the severity spectrum of symptoms correlated to long-term outcome data, prognostic markers may be identified that will aid in future counseling of parents. Imperative to the success of a pilot universal screening program is also the development of protocols for systematic data-collection. Furthermore, the psychological impact of screening on parents and affected children should be evaluated and considered in the overall benefit analysis.

As Griffiths points out, we are stuck in a classic 'Catch-22' situation in which screening is not advocated due to the lack of treatment options, yet lack of screening prevents adequate randomization necessary for appropriately exploring treatment efficacy.[118] Large screening programs could provide the foundation for future research into new management practices/therapies and stimulate the development of evidence-based guidelines in the care of infants with cCMV infection.

Specimen for universal screening

With the aim of universal screening, future studies should examine the most suitable specimen for screening and further optimize the methodologies and means for high-throughput testing.

Both urine and saliva contain high viral loads, making them ideal candidates; however, both would require implementation of new collection, transport and processing facilities. Ideally, DBS CMV-PCR would be used based on the existing infrastructure of collecting DBS cards in the first week of life. The methodology for this assay should be further optimized taking into consideration the numerous confounders (differences in populations screen (i.e. selected versus unselected), filter paper size/

specimen volume, DNA extraction method, PCR protocols) that impact sensitivity. Recent studies of unselected infants[69,119] suggest that there is capacity for this assay to be implemented. As suggested by Koontz et al., with sufficient market demand, existing sensitive DBS CMV-PCR protocols could be further developed for automated, high-throughput testing.[68]

Awareness of cCMV infections

Future research should focus on education and counselling of pregnant women on hygienic measures with the aim of preventing CMV transmission to the fetus. Expecting mothers should know how to protect themselves and their unborn baby from infectious agents, similar to advice given on measures to avoid toxoplasmosis and listeria infection.[120] Educational platforms should stimulate the general public health concern of cCMV infections, which adversely affect more children than any other childhood diseases or syndromes.[121] With the addition of information on cCMV infections into obstetric and pediatric residency- and continued education programs, more awareness and knowledge will be generated amongst health care professionals.

Definition of cCMV infection

Imperative to the future study of cCMV infection, an internationally accepted definition of the symptoms associated with cCMV infection, should be formulated. The comparison of international studies is frequently hampered by differences in criteria of symptomatic CMV infection.[122] Developing such a consensus on symptoms will allow for better comparative studies in the future. With a clear definition and data from universal screening programs the clinical relevance of mild-moderate, self-limiting symptoms could be further evaluated.

Impact and definition of non-primary cCMV infections

Through our small case series in Chapter 2 and amongst the literature[123,124] we have demonstrated and confirmed the impact of non-primary infections on fetal development and the pitfalls regarding the diagnosis in mothers. Future research should focus on furthering our understanding of the serological profiles (differentiating between reinfection and reactivation) of non-primary infections and the relative risk posed to the developing fetus throughout gestation. A better understanding of the pathophysiological mechanisms and immune correlates involved in vertical transmission may also guide the development of future vaccination strategies in seropositive pregnant women.

Vaccine development

Ideally, CMV would be eradicated through the use of a universal vaccine. Promising advances in this field have been made using live-attenuated virus or subunit components of CMV. Future research should focus on furthering our understanding of the immune correlates of protection against CMV and combining strong immunogens to generate durable and protective immune responses.

Antiviral therapy and the risk of resistance

In the transplant population, the development of viral resistance as a result of prolonged treatment with antivirals such as ganciclovir is not uncommon.[125] Although the development of resistance amongst infants with cCMV infection is limited to incidental reports,[126] antiviral resistance may increasingly occur with the extension of (val)ganciclovir treatment regimes. The gold standard for identification of viral resistance is the plaque reduction assay apt at identifying novel mutations.[125] This method however, is labor intensive and time consuming. Genotypic assays are rapid and automated, however only detect known mutations. Future research should focus on an assay that is both fast and sensitive and able to detect novel mutations.

Sequelae in (subgroups of preterm infants with) pCMV infections

With increasing possibilities to sustain life in extremely preterm infants (GA<24 weeks), the short- and long term sequelae of a pCMV infection in these infants remains to be determined and should be subject of a future study. In parallel, the scope of symptoms as a result of a pCMV infection in this population should be further defined to better characterize symptomatic pCMV infection. As we have demonstrated, neurodevelopment of preterm infants is not affected by a pCMV infection until six years of age. In light of the negative impact on cognitive development amongst adolescent subjects with pCMV infection demonstrated by the German research group, it would be of interest to examine the cognitive performance of our cohort again at a later stage of development.

Treatment strategies for pCMV infection

Untreated breastmilk has numerous nutritional and immunological benefits for the infant and also positively affects neurodevelopment.[114] As we have shown, pre-treatment of breastmilk to prevent CMV transmission appears to be unnecessary in the general NICU population. Extreme preterm infants (GA <24 weeks) with co-morbidities, depending on their clinical stability may benefit from pre-treatment of breastmilk on a case-by-case basis. Future research should focus on optimizing methods of pre-treatment strategies to enable fast and complete elimination of CMV in breastmilk without affecting the vital nutritional and immunological properties. Furthermore, severe symptomatic pCMV disease (e.g. in infants with severe co-morbidities) should be analyzed to determine the added value of antiviral treatment with respect to mortality and long term outcome.

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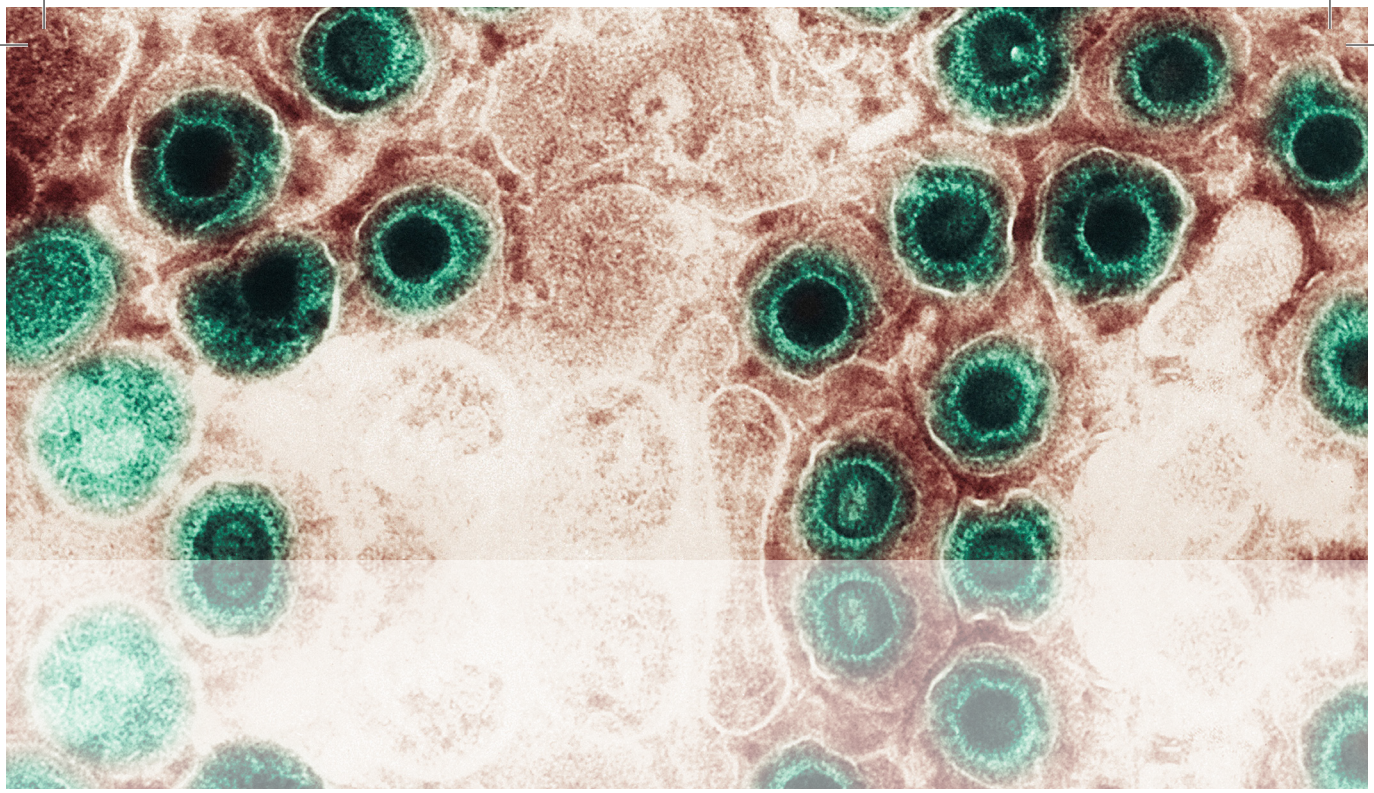
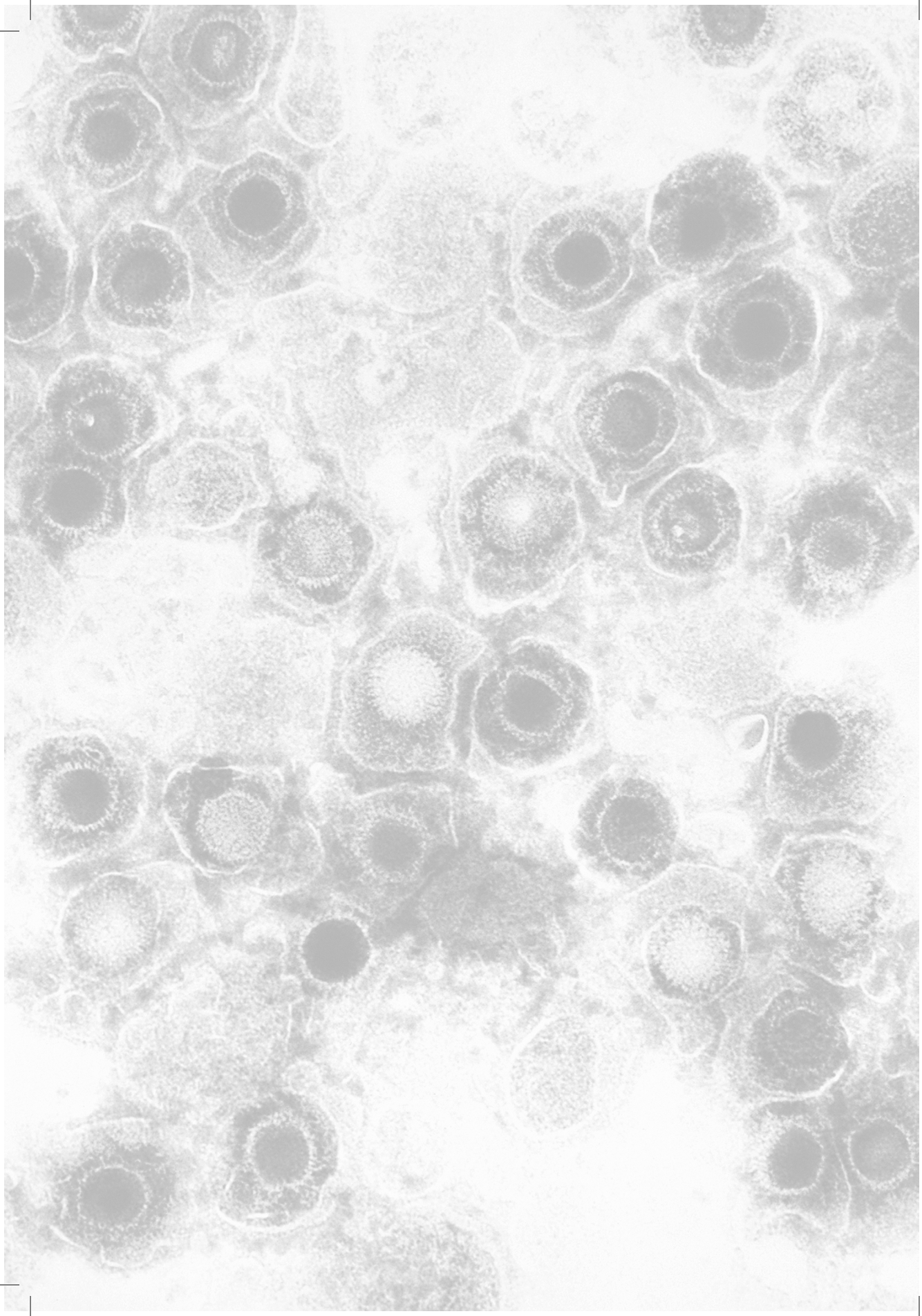


Nederlandse samenvatting
Lists of publications and co-authors
Dankwoord
Curriculum Vitae



Nederlandse samenvatting

Cytomegalovirus

Het cytomegalovirus (CMV) is een veel voorkomend, dubbelstrengs DNA virus en het grootste lid van de familie herpesvirussen. CMV is opgebouwd uit drie compartimenten: een icosahedrale nucleocapside die het virale genoom bevat, omgeven door een eiwitrijke tegument-laag en van buiten ingekapseld door een lipiden buitenenvelop (Figuur 1, pagina 2). Kenmerkend voor alle infecties met herpesvirussen is levenslange latente aanwezigheid binnen de gastheer afgewisseld met intermitterende perioden van virale reactivatie, gevolgd door uitscheiding van virusdeeltjes. Primaire infectie met CMV kan tot stand komen door contact met lichaamsvloeistoffen, waaronder speeksel, bloed, urine, vruchtwater en moedermelk.

Het exacte mechanisme van de reactivaties is nog niet helemaal duidelijk, maar gebeurt hoogstwaarschijnlijk tijdens perioden van inflammatie. Voor CMV geldt dat men gereïnfecteerd kan raken na contact met een andere stam dan waarmee de primaire infectie heeft plaatsgevonden. Reactivatie en reïnfectie zijn ook bekend als 'non-primaire CMV infectie'.

CMV infecties kunnen op vijf verschillende manieren plaatsvinden:

1. Congenitale CMV infectie, waarbij CMV van de moeder op het ongeboren kind via de placenta (moederkoek) wordt overgedragen;
2. Perinatale CMV infectie (infectie binnen een maand na de geboorte);
3. Postnatale CMV infectie (infectie binnen de eerste 12 maanden na de geboorte), waarbij het kind CMV oploopt door contact met geïnfecteerde afscheiding in het geboortekanaal of een andere geïnfecteerde lichaamsvloeistof (meestal door borstvoeding);
4. Infectie van een immunocompetente gastheer zich uitend in mononucleose of een subklinische infectie;
5. Infectie van immuungecompromitteerde personen (zoals transplantatiepatiënten) die vaak gepaard gaat met CMV ziekte

Congenitale (aangeboren) CMV infectie

Een congenitale CMV infectie (cCMV) is de meest voorkomende aangeboren virusinfectie wereldwijd. Moeders die nog nooit in aanraking zijn geweest met CMV (CMV seronegatief) en voor het eerst een CMV infectie oplopen tijdens de zwangerschap (primaire infectie), dragen het virus vaker over op hun ongeboren kind dan moeders die al voor de zwangerschap CMV hebben opgelopen (CMV seropositief) en een reactivatie of reïnfectie doormaken. In Nederland wordt ongeveer 1 op de 200 pasgeborenen (0.5%) met een cCMV infectie geboren, waarvan ongeveer 10-13% symptomen bij de geboorte vertoont en de rest asymptomatisch is. De symptomen van een cCMV infectie kunnen variëren van mild tot ernstig en levensbedreigend. Vooral pasgeborenen die geïnfecteerd zijn als gevolg van een maternale primaire infectie in het eerste trimester lopen risico op (ernstige) afwijkingen, zoals microcefalie, aanlegstoornissen van de hersenen en ernstig gehoorverlies. Uit de symptomatische groep zal ongeveer 60% langetermijngevolgen ontwikkelen zoals cerebrale parese, epilepsie, visusstoornissen, gehoorverlies en/of psychomotore retardatie. Maar ook in de bij de geboorte asymptomatische groep zal alsnog ongeveer 9-18% langetermijngevolgen ontwikkelen, meestal in de vorm van gehoorverlies.

Er bestaat op dit moment geen effectieve behandeling. Behandeling met het antivirale middel (val)ganciclovir kan bij symptomatische kinderen de kans op gehoorverlies verkleinen. Het vaccineren van zwangere vrouwen zou een goede oplossing zijn voor het voorkomen van een cCMV infectie, maar er is tot op heden nog geen volledig beschermend vaccin ontwikkeld. Preventieve maatregelen tijdens de zwangerschap die de hygiëne verbeteren (kleine kinderen niet op de mond kussen, geen bekers of bestek delen met peuters) zijn op dit moment het meest effectief om een infectie met CMV te voorkomen.

Perinatale of postnatale (verworven) CMV infectie

Perinatale of postnatale CMV (pCMV) infectie komt vaak voor onder pasgeborenen (mediane incidentie ~20%). Moeders die CMV seropositief zijn, scheiden in 96% van de gevallen CMV uit in hun borstvoeding. Dit leidt in 37% van de gevallen tot een infectie bij het kind. Men spreekt dan ook over een verworven CMV infectie. Risicofactoren voor het oplopen van een pCMV infectie zijn moeders met een niet-Westerse etniciteit aangezien de prevalentie van CMV seropositiveit onder deze moeders hoger is. Bij pasgeborenen die bij een voldragen zwangerschapsduur zijn geboren verloopt een pCMV infectie altijd asymptomatisch. Bij kinderen die (veel) te vroeg worden geboren (<32 weken, prematuur geboren zuigelingen) is een pCMV infectie meestal asymptomatisch en zijn symptomen indien deze wel ontstaan over het algemeen mild en van tijdelijke aard. Het meest voorkomende symptoom is een 'CMV-achtige sepsis', in andere woorden: sepsis-achtige symptomen en trombocytopenie. Op cerebrale echografie uitgevoerd bij prematuur geboren zuigelingen met een pCMV infectie op de leeftijd van een voldragen zwangerschap (40 weken postmenstruele leeftijd) wordt vaak lenticulostriatale vasculopathie (LSV) gezien. Gehoorsverlies lijkt niet voor te komen als gevolg van een pCMV infectie. Over de langetermijngevolgen bestaat onduidelijkheid. De ontwikkeling in de eerste twee levensjaren lijkt niet afwijkend te zijn. Recent laten een aantal Duitse studies op basis van kleine aantallen, significante verschillen zien in de cognitieve ontwikkeling tussen ex-prematuur geboren zuigelingen met een pCMV infectie en niet geïnfecteerde kinderen in de schoolgaande leeftijd en adolescentie.
In het merendeel van de gevallen is behandeling niet geïndiceerd en gaan de symptomen vanzelf weer over. Incidenteel wordt er in de literatuur beschreven dat (val) ganiclovir gegeven wordt bij prematuur geboren zuigelingen met een ernstige sepsisachtig ziektebeeld en/of bij ernstige comorbiditeiten op basis van de prematuriteit. Vanwege het risico op een symptomatische pCMV infectie in deze kwetsbare groep en de onzekerheid over de langetermijngevolgen, worden er in sommige landen preventieve maatregelen genomen om transmissie door borstvoeding te voorkomen. Dit gebeurt door invriezen of pasteuriseren van de borstvoeding. Beide technieken hebben hun voor- en nadelen. Invriezen heeft als voordeel dat de natuurlijke immuun- en voedingsvoordelen van borstvoeding niet beïnvloed worden, hierbij wordt CMV echter niet compleet geëlimineerd. Pasteurisatie elimineert CMV wel volledig, maar dit gaat door denaturatie van eiwitten ten koste van de voordelen van de borstvoeding. Met name bij prematuur geboren zuigelingen is het geven van onbehandelde borstvoeding essentieel voor de groei én het voorkomen van comorbiditeit ten gevolge van de prematuriteit.

Dit proefschrift

In het eerste deel van dit proefschrift hebben we gekeken naar de valkuilen bij het diagnosticeren van een cCMV infectie tijdens de zwangerschap. Verder hebben we de ziektelast in Nederland bepaald en daarnaast in verschillende landen de actuele klinische standaarden geëvalueerd wat betreft herkenning en behandeling van kinderen met een cCMV infectie. Tenslotte hebben we de bevindingen op cerebrale beeldvorming na de geboorte gecorreleerd aan het moment dat de moeder een CMV infectie opliep tijdens de zwangerschap en de ontwikkeling van het kind.

In het tweede deel hebben we gekeken naar prematuur geboren zuigelingen met een pCMV infectie. We hebben bestaande diagnostische mogelijkheden geëvalueerd om een snelle diagnose te kunnen stellen. Verder hebben we bevindingen op cerebrale beeldvorming beschreven en de langetermijnuitkomsten van geïnfecteerde kinderen onderzocht.

Deel I — Congenitale CMV infectie

In hoofdstuk 2 beschrijven we vijf gevallen waarbij foetale echografische afwijkingen worden gezien in het tweede en derde trimester die een cCMV infectie suggereren. Op de routine echografie bij 20 weken zwangerschapsduur bleken alle foetussen cerebrale en extracerebrale afwijkingen te vertonen. Een cCMV infectie werd in alle gevallen verworpen op basis van een negatieve CMV-IgM bij de moeder ten tijde van het ontdekken van de foetale afwijkingen. In alle gevallen werd door de aanwezigheid van klinische symptomen of foetale obductie bevindingen na de geboorte, de diagnose cCMV infectie echter bevestigd. Vervolgens werd het maternale serum uit het eerste trimester retrospectief op CMV getest, waarbij er in het eerste geval een klassieke primaire infectie met een positieve CMV-IgM gezien werd. In het tweede geval was het type infectie niet te bepalen, omdat het eerste trimester serum niet meer beschikbaar was. In het derde geval ging het waarschijnlijke om een primaire infectie en in de laatste twee gevallen om non-primaire infecties van de zwangere. Deze serie laat de valkuilen zien van het interpreteren van maternale CMV serologie tijdens de zwangerschap en benadrukt het belang van het inzetten van meerdere diagnostische tests voor het aantonen dan wel uitsluiten van een cCMV infectie. In figuur 1 van hoofdstuk 9 (pagina XX) geven we onze aanbevelingen ten aanzien van de diagnostiek bij echografische foetale afwijkingen die op een cCMV infectie zouden kunnen wijzen. Als tijdens de zwangerschap verdenking op een cCMV infectie bestaat, of het kind wordt geboren met symptomen van een cCMV infectie, moet de urine van het kind ≤2-3 weken na de geboorte op CMV getest worden om de infectie te kunnen bevestigen.

In hoofdstuk 3 hebben we de bevindingen op cerebrale beeldvorming na de geboorte gecorreleerd aan het moment dat de moeder een CMV infectie opliep tijdens de zwangerschap (i.e. eerste, tweede of derde trimester) en de ontwikkeling van het kind. Verder hebben we de toegevoegde waarde van cerebrale MRI onderzocht (naast cerebrale echografie) op het detecteren van hersenafwijkingen ten gevolge van CMV.

Kinderen die een cCMV infectie kregen in het eerste trimester van de zwangerschap, hadden vaker symptomen bij de geboorte en ook ernstiger hersenafwijkingen. Als gevolg daarvan hadden zij een afwijkende ontwikkeling bij een leeftijd van 16-18 maanden. Van de groep zonder hersenafwijkingen bij de geboorte had iedereen een normale ontwikkeling bij 16-18 maanden, ondanks het feit dat sommige kinderen klinische symptomen hadden bij de geboorte. Infecties in het tweede en derde trimester van de zwangerschap hadden geen negatief effect op de ontwikkeling van het kind. Verder hebben we aangetoond dat bij de aanwezigheid van MRI afwijkingen ook altijd afwijkingen op de cerebrale echografie te zien zijn. Bovendien bleek MRI effectiever in het aantonen van migratiestoornissen (aanlegstoornis van de hersenschors) in vergelijking met cerebrale echografie. Cerebrale echografie was effectiever in het aantonen van calcificaties (i.e. lenticulostriatale vasculopathie) en germinolytische cysten.

In hoofdstuk 4 hebben we een online vragenlijst verstuurd aan een geselecteerde groep internationale experts betrokken in de moederkindzorg. Met behulp van deze vragenlijst hebben we de klinische standaard en effectiviteit van herkenning en behandeling van kinderen met een cCMV infectie geëvalueerd. Gelijktijdig hebben we een nationale cCMV registratie opgezet om de ziektelast in Nederland te bepalen.
Het merendeel van de respondenten van de vragenlijsten was neonatoloog met een speciale interesse in neonatale neurologie. Over het algemeen viel op dat er weinig overeenstemming was in het screeningsbeleid, de symptoomherkenning, de neurologische beeldvorming, de behandeling en de reguliere follow-up. Zo gaf bijvoorbeeld 16% van de respondenten aan het symptoom gehoorverlies niet als aanleiding om CMV diagnostiek bij het kind in te zetten. Ook wat betreft de indicatie voor behandeling gaven sommige respondenten naast symptomatische ook asymptomatische kinderen te behandelen, alhoewel er geen klinische studies zijn die de werkzaamheid van antivirale behandeling op langetermijngevolgen bij asymptomatische kinderen hebben onderzocht.
In de nationale registratie is er in totaal over een periode van drie jaar slechts 20% van het vooraf verwachte aantal kinderen geregistreerd. Het merendeel van de geregistreerde kinderen was symptomatisch bij de geboorte en had afwijkingen van het centrale zenuwstelsel. Ook binnen Nederlandse ziekenhuizen waren er verschillen in de behandeling van geïnfecteerde kinderen. Dit wijst op weinig uniformiteit in de aanpak van behandeling.

Discussie — deel I

Ons onderzoek uit deel I van dit proefschrift maakt duidelijk dat prenatale diagnostiek naar CMV lastig is en soms leidt tot het onterecht verwerpen van de diagnose, er weinig uniformiteit bestaat in de postnatale CMV diagnostiek en er grote verschillen bestaan in behandelbeleid.

Afwijkingen in het brein zijn een sterke voorspeller van een slechte ontwikkeling. Een primaire infectie met CMV in het begin van de zwangerschap bij de moeder levert het grootste risico op voor het ontwikkelen van ernstige hersenafwijkingen, zoals we bevestigd hebben in hoofdstuk 3. Alhoewel minder frequent kunnen non-primai-

re infecties (reactivaties of reïnfecties) ook ernstige afwijkingen bij het ongeboren kind veroorzaken (hoofdstuk 2). Het is dus van belang om bij kinderen met een cCMV infectie na de geboorte het brein te onderzoeken door cerebrale echografie en zonodig aanvullend met een cerebrale MRI. Het merendeel van de respondenten in hoofdstuk 4 gaf aan dat alle kinderen (symptomatisch en asymptomatisch) als onderdeel van de standaard zorg bij cCMV infecties een schedelecho kregen. Het uitvoeren van cerebrale beeldvorming is belangrijk om de prognose van het kind in te kunnen schatten zodat de ouders volledig over de prognose van hun kind geïnformeerd kunnen worden.

Ondanks het feit dat we in hoofdstuk 4 maar een selecte groep medische experts hebben geïncludeerd en de resultaten niet gegeneraliseerd mogen worden, gaf dit in combinatie met de lage inclusie van de nationale registratie de indruk dat kinderen met een cCMV infectie waarschijnlijk niet goed herkend worden. Zo koos 16% bij de vragen over symptoomherkenning niet voor gehoorverlies als reden om op CMV te testen en koos slechts 35% van de respondenten bij alle negen CMV geassocieerde symptomen voor diagnostiek. Het probleem bij de diagnostiek van cCMV infecties is dat het merendeel van de symptomen aspecifiek zijn voor een CMV infectie en dat de symptomen vaak van tijdelijke aard zijn en vanzelf weer overgaan. Echter, ook de groep kinderen met mildere symptomen en de asymptomatische kinderen kunnen langetermijngevolgen ontwikkelen, zoals gehoorverlies.

Om cCMV infecties te bestuderen zijn er in het verleden al een aantal registraties opgestart in verschillende landen, maar ook deze studies beschrijven lage inclusies. Dit kan komen doordat kinderen niet goed herkend worden of omdat artsen er niet aan toe komen om geïnfecteerde kinderen te melden. Op basis van de resultaten van de reeds gepubliceerde registratiestudies en onze eigen bevindingen in de Nederlandse registratiestudie concluderen we dat (nationale) registraties niet effectief genoeg zijn om het ziektebeeld en de ziektelast van cCMV infecties te bestuderen. Een mogelijke oplossing om de problemen van registraties te voorkomen, is door kinderen vlak na de geboorte te screenen op CMV. Hierdoor kunnen alle kinderen die het risico lopen op het ontwikkelen van langetermijngevolgen van een cCMV infectie, tijdig geïdentificeerd worden. Bij een vroege diagnose zou er snel therapeutisch ingegrepen kunnen worden als er zich afwijkingen in de ontwikkeling voordoen. Ook zouden kinderen die aan de criteria voldoen voor therapie tijdig met het antivirale middel (val)ganciclovir behandeld kunnen worden. Vooral bij gehoorverlies is het vroege detecteren en behandelen uiterst belangrijk voor een normale spraak- en taalontwikkeling.

De voordelen van universele screening zouden eerst bestudeerd moeten worden in een pilotstudie. In hoofdstuk 9, figuur 3 doen we een voorstel hoe een dergelijke studie eruit zou kunnen zien in Nederland. Het screenen van kinderen zou met urine en met de hielprikkaart (Guthrie kaart) gedaan kunnen worden binnen drie weken na de geboorte. Idealiter vindt universele CMV screening plaats door de standaard neonatale hielprik screening. De bestaande analysemethoden hiervoor moeten echter nog verder worden geoptimaliseerd, zodat alle CMV positief bevonden kinde-

ren juist geïdentificeerd worden en geen negatieve kinderen als positief bevonden worden. Alle positief bevonden kinderen worden vervolgens door een kinderarts onderzocht die het klinische beeld in kaart brengt door lichamelijk onderzoek en aanvullende diagnostiek. Symptomatische kinderen worden vervolgens op basis van de CMV-richtlijn van de Nederlandse Vereniging voor Kindergeneeskunde behandeld en opgevolgd. Omdat asymptomatische kinderen minder risico lopen op het ontwikkelen van langetermijngevolgen van een cCMV infectie is follow-up via het consultatiebureau voldoende. Wel moeten gehooronderzoeken gedaan worden bij bijvoorbeeld een leeftijd van zes manden, 12 maanden en dan jaarlijks tot zes jaar. Als er een afwijkende ontwikkeling geconstateerd wordt zal moeten worden doorverwezen naar een kinderarts. Een ander belangrijk onderdeel van een dergelijke studie is het verzamelen van data ten aanzien van de symptomen en langetermijngevolgen van een cCMV infectie.

Deel II — Postnatale CMV infectie

In hoofdstuk 5 hebben we een overzicht gegeven van de huidige kennis op het gebied van pCMV infecties bij prematuur geboren zuigelingen (kinderen geboren bij een zwangerschapsduur <32 weken). Verder hebben we een opsomming van factoren gegeven die in deze kwetsbare groep een ernstig klinisch beloop zouden kunnen voorspellen. Belangrijk is dat het merendeel van de prematuur geboren zuigelingen met een pCMV infectie asymptomatisch blijft. De belangrijkste twee risicofactoren op het ontwikkelen van symptomen zijn extreme vroeggeboorte en een te laag geboortegewicht. Vooral extreem prematuur geboren zuigelingen (geboren bij <28 weken) en/of kinderen met een te laag geboortegewicht (<1500 gram) hebben een grotere kans op het ontwikkelen van symptomen zoals een CMV-sepsisachtig syndroom. Het is niet duidelijk of het ontwikkelen van symptomen een directe oorzaak is van de CMV infectie alleen of dat symptomen zich ontwikkelen als gevolg van de CMV infectie in een kind met een onderontwikkeld immuunsysteem in samenhang met veel comorbiditeiten door de prematuriteit.

In hoofdstuk 6 hebben we bij prematuur geboren zuigelingen het screenen van urine CMV-PCR vergeleken met het screenen van speeksel CMV-PCR voor het aantonen van een pCMV infectie. Het verzamelen van speeksel is makkelijker en sneller dan het opvangen van urine en zou een praktisch alternatief kunnen zijn. Tijdens een bezoek aan de follow-up polikliniek wanneer de kinderen de leeftijd hadden van een voldragen zwangerschap (40 weken postmenstruele leeftijd) werd er een urine en een speeksel monster afgenomen en getest middels CMV-PCR. Bij 18% van de kinderen werd een positieve uitslag gevonden middels urine CMV-PCR en bij 16.1% middels speeksel CMV-PCR. In totaal heeft de speeksel CMV-PCR vijf kinderen gemist die positief waren met de urine CMV-PCR. Anderzijds werd één kind bij de speeksel CMV-PCR positief bevonden, maar negatief bij de urine CMV-PCR. De gevoeligheid van de speeksel CMV-PCR werd beter naarmate er alleen kinderen met symptomen geïncludeerd werden. Het blijkt dat screenen op postnatale CMV met speeksel minder effectief is dan urine, maar om een snelle diagnose te stellen bij een kind met symptomen van een pCMV infectie zou speeksel een praktisch alternatief of eerste test kunnen zijn.

In hoofdstuk 7 hebben we de integriteit van de witte stof in de hersenen geanalyseerd bij 21 prematuur geboren kinderen met een pCMV infectie en 61 niet geïnfecteerde prematuur geboren kinderen. Door gebruik te maken van diffusion tensor imaging (DTI), waarbij de diffusie snelheid en richting van water in het brein gemeten wordt als maat voor hersenschade, hebben we onderzocht of er mogelijk verschillen zijn in de witte stof door een pCMV infectie. Bij eerdere studies in prematuur geboren zuigelingen werden witte stofafwijkingen op een voldragen leeftijd geassocieerd met een slechtere ontwikkeling.

We hebben in deze studie met behulp van DTI drie regio's in de witte stof (frontaal, pariëtaal, occipitaal) geanalyseerd. De metingen werden vervolgens uitgedrukt in verschillende parameters waaronder een gemiddelde diffusie (MD) en fractionele anisotropie (FA). In vergelijking met de controlegroep werd er bij de groep van geinfecteerde kinderen een significant lagere FA gezien in de occipitale witte stof. Dit kan wijzen op kleine structurele afwijkingen in de witte stof. Om de significantie van deze bevindingen verder uit te zoeken hebben we vervolgens naar de ontwikkelingsuitkomst van beide groepen gekeken bij een leeftijd van 16 maanden, gecorrigeerd voor de prematuriteit. Er waren geen verschillen tussen beide groepen, wat aangeeft dat er in ieder geval tot deze leeftijd geen consequenties zijn van deze subtiele occipitale afwijkingen.

In hoofdstuk 8 hebben we de korte- en langetermijnontwikkeling van prematuur geboren zuigelingen met een pCMV infectie op verschillende (gecorrigeerde) leeftijden tot zes jaar onderzocht. Uit de literatuur blijkt dat de kortetermijnontwikkelingvan kinderen met een pCMV infectie niet afwijkend is. In meer recente studies werd er echter een slechtere cognitieve ontwikkeling gesuggereerd rond de leeftijd van acht jaar en in de adolescentie. Om meer duidelijkheid te verkrijgen over de mogelijke gevolgen hebben we de ontwikkeling van een groot cohort van prematuur geboren zuigelingen op drie leeftijden (16 maanden, 24-30 maanden, 5-6 jaar) onderzocht.
Bij 16 maanden zijn beide groepen getest met de Griffiths Mental Development Scales (GDMS), waarbij geen verschillen werden gezien behalve dat geïnfecteerde kinderen een beter resultaat behaalden op de grofmotorische schaal van de GMDS. Op een leeftijd van 24-30 maanden waren er geen significante verschillen tussen beide groepen die werden getest met behulp van de GMDS of de Bayley Scales of Infant and Toddler Development-III (BSITD-III). Geïnfecteerde kinderen begonnen echter wel significant vroeger met zelfstandig lopen dan de controlegroep. Multivariabele regressie analyse liet zien dat dit te maken heeft met een niet-Westerse etniciteit van de moeder. Op de leeftijd van zes jaar zijn de kinderen getest met de Wechsler Preschool and Primary Scale of Intelligence-III (WPPSI-III), wat een cognitieve ontwikkelingstoets is. Hierbij behaalden geïnfecteerde kinderen normale resultaten op alle domeinen van de toets, maar gemiddeld genomen wel lager dan de controlegroep. Alleen op het domein van verbale vaardigheden was dit significant verschillend. Multivariabele regressie toonde aan dat dit door een niet-Westers maternale etniciteit en door maternaal opleidingsniveau kwam. CMV status had geen significante invloed. Verdere analyses lieten geen verschillen zien tussen symptomatische en asymptomatische kinderen. Motorische vaardigheden werden aan hand van de Movement As-

sessment Battery for Children-II (MABC-II) getoetst. Er waren geen verschillen tussen beide groepen. Op de leeftijd van zes jaar hebben we ook het gehoor geëvalueerd bij geïnfecteerde kinderen. Er was geen sprake van perceptief gehoorverlies.

Discussie — deel II

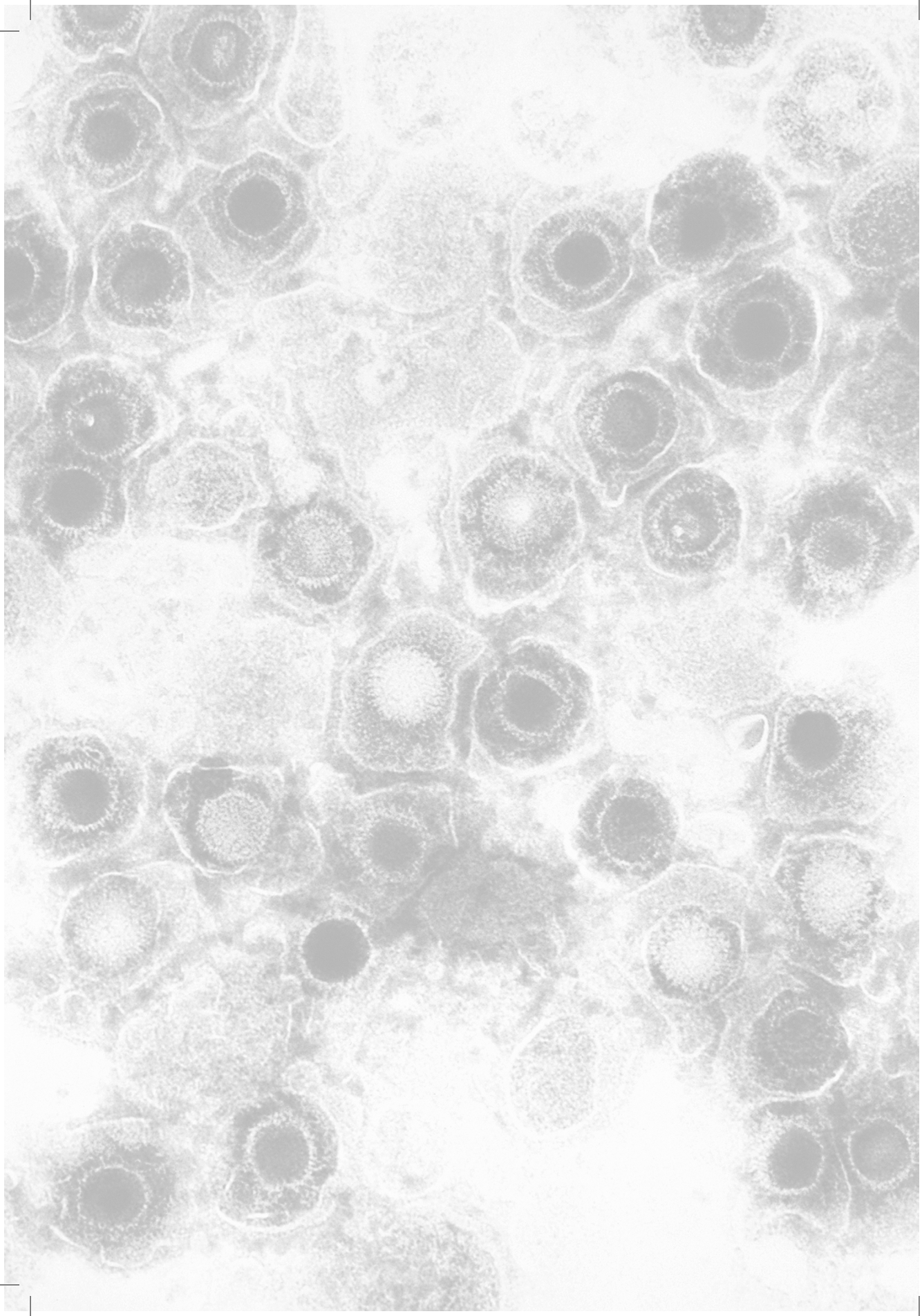
De ontwikkeling van prematuur geboren zuigelingen met een pCMV infectie blijft een punt van discussie, vooral omdat reeds gepubliceerde studies tegenstrijdige resultaten geven. Er zijn vooral zorgen omdat een infectie bij een extreme prematuur mogelijk vergelijkbaar is met een cCMV infectie in het derde trimester van de zwangerschap. In dit proefschrift hebben we een groot cohort van prematuur geboren zuigelingen prospectief gevolgd tot de leeftijd van zes jaar om de mogelijke (lange termijn) gevolgen van een pCMV infectie te onderzoeken en om te beoordelen of preventieve maatregelen noodzakelijk zijn om een CMV infectie kort na de geboorte te voorkomen.

Bij kinderen met een cCMV infectie is het voorkomen van hersenafwijkingen een sterke voorspeller voor een afwijkende ontwikkeling, zoals we hebben laten zien in hoofdstuk 3. Door aanvullende diagnostiek middels DTI, lieten we zien dat postnataal geïnfecteerde kinderen vaker een significant lagere FA (structurele afwijking in de witte stof) hadden in de occipitale witte stof (hoofdstuk 7). De significantie van deze bevindingen is niet duidelijk. Ernstige witte stof schade in de occipitaalkwab zou kunnen leiden tot visusstoornissen, maar cerebrale blindheid is nog nooit eerder beschreven als gevolg van een pCMV infectie. In ons cohort hebben geïnfecteerde kinderen geen uitgebreide visus test gehad, maar ze behaalden wel een beter resultaat op de balvaardigheden schaal van de MABC-II bij zes jaar (hoofdstuk 8). Bij deze toets moet het kind een voorwerp nauwkeurig gooien en vangen, een taak waarbij men een goed gezichtsvermogen moet hebben.

Alhoewel het beloop van een pCMV infectie meestal asymptomatisch is, blijkt de groep van extreem prematuur geboren zuigelingen en neonaten met een te laag geboortegewicht risico te lopen op het ontwikkelen van symptomen. In ons cohort hebben vier van de 74 geïnfecteerde kinderen (5%) symptomen ontwikkeld (hoofdstuk 8). Dit percentage komt overeen met wat er in de literatuur gepubliceerd is (mediaan incidentie: 3%). Alle symptomen waren mild en van tijdelijk aard en antivirale behandeling was niet noodzakelijk. Recentelijk werd er een hoog percentage (48%) aan symptomatische pCMV infectie beschreven bij kinderen die bij 22-24 weken zwangerschapsduur geboren werden. In Nederland wordt een actief neonatologisch beleid vanaf 24 weken zwangerschapsduur aanbevolen. Vandaar dat deze kinderen niet in ons cohort werden geïncludeerd. Over de langetermijnontwikkeling van deze specifieke groep extreme prematuur geboren zuigelingen kunnen wij geen uitspraken doen. Ons onderzoek toont aan dat voor kinderen die na 24 weken en voor 32 weken zwangerschapsduur geboren zijn, de langetermijnontwikkeling niet negatief beïnvloed werd door een pCMV infectie tot een leeftijd van zes jaar.

Een Duitse studie heeft recentelijk aangetoond dat prematuur geboren zuigelingen met een pCMV infectie op een adolescente leeftijd een slechtere cognitieve uitkomst hadden dan de controle groep 1,2. Over het algemeen is het lastig om ontwikkeling zorgvuldig te onderzoeken door de vele factoren die de uitkomst kunnen beïnvloeden en door de vaak ondervonden 'loss to follow-up'. Een interessante overeenkomst tussen de Duitse studie en onze studie is dat geïnfecteerde kinderen over het algemeen een lager resultaat hadden bij cognitieve taken. Terwijl de Duitse onderzoekers dit aan CMV status wijden, hebben we deze associatie in ons grotere cohort niet kunnen vaststellen, om dat dit onafhankelijk gecorreleerd bleek te zijn met maternale etniciteit en opleidingsniveau van de moeder, factoren waarvoor niet gecorrigeerd werd in de Duitse studie.

Onze studie bij een groot cohort prematuur geboren zuigelingen laat zien dat symptomen als gevolg van een pCMV infectie weinig voorkomen en dat bij de geinfecteerde kinderen geen antivirale behandeling nodig is. Zoals eerder benoemd wordt er incidenteel beschreven dat (val)ganciclovir gegeven wordt aan ernstig en levensbedreigend zieke kinderen met een pCMV infectie. Er zijn echter nog geen gerandomiseerde studies uitgevoerd die de werkzaamheid van (val)ganciclovir voor deze indicatie onderzocht hebben. Tot er meer bewijs is voor de werkzaamheid van antivirale medicatie, moet het geven daarvan per individu bepaald worden. Vooral bij prematuur geboren zuigelingen is het geven van moedermelk sterk aanbevolen vanwege de immunologische voordelen en de rijke voedingswaarde. Aangezien een pCMV infectie geen nadelig effect had op de langetermijnontwikkeling, is er geen indicatie om potentieel geïnfecteerde moedermelk voor te behandelen. Bij de groep van extreme prematuur geboren zuigelingen (zwangerschapsduur <24 weken) of kinderen met veel en ernstige comorbiditeiten kan overwogen worden om de moedermelk wel voor te behandelen tot er een klinische stabiele situatie is.



List of publications
List of co-authors and their affiliations

List of publications

Gunkel J*, van der Knoop BJ *, Nijman J, de Vries LS, Manten GTR, Nikkels PGJ, Murk JL, de Vries JIP, Wolfs TFW. Congenital cytomegalovirus infection in the absence of maternal CMV-IgM antibodies. E-publication ahead of print, Fetal Diagn Ther. 2017 Mar 4

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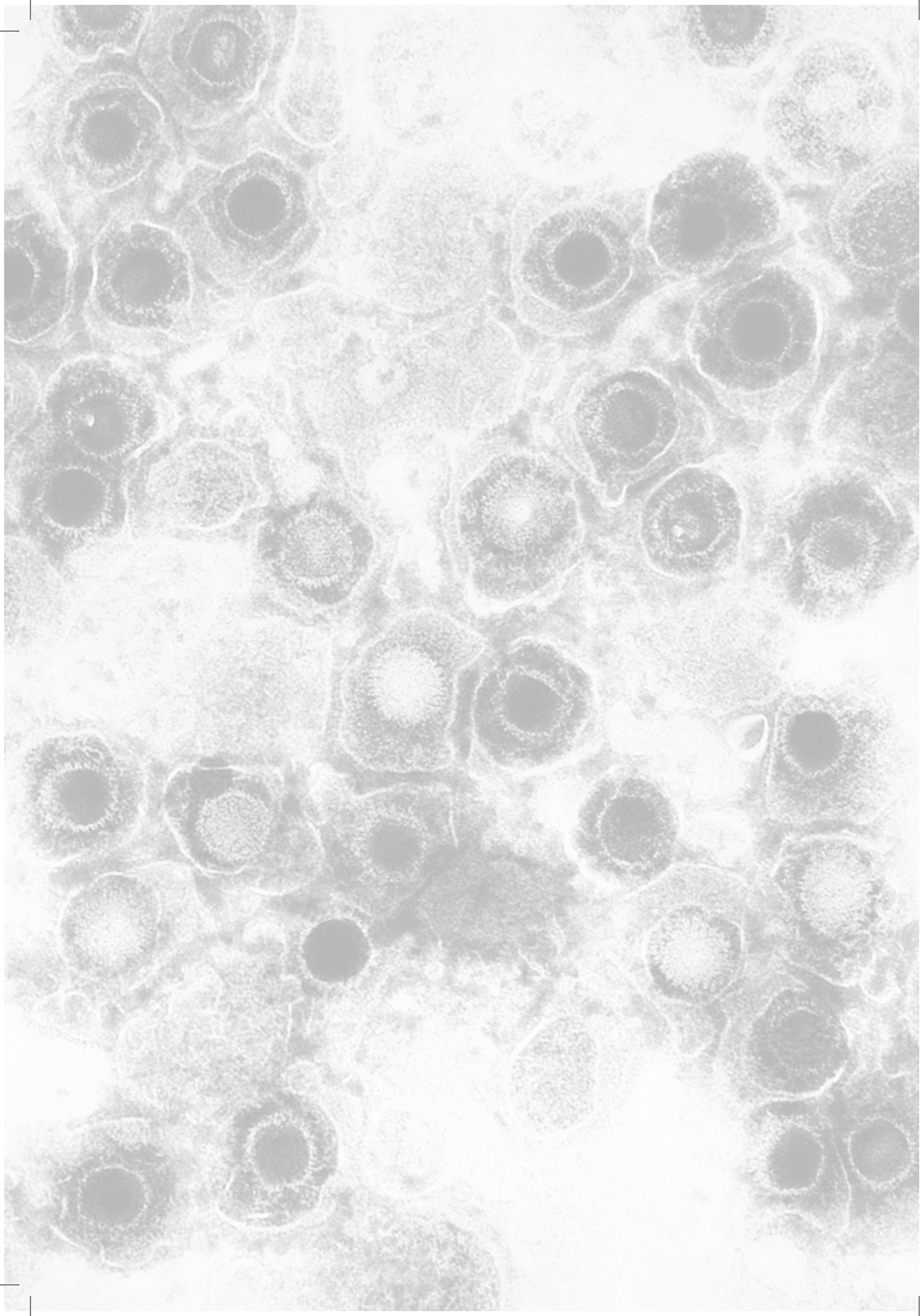

Dankwoord

Dankwoord

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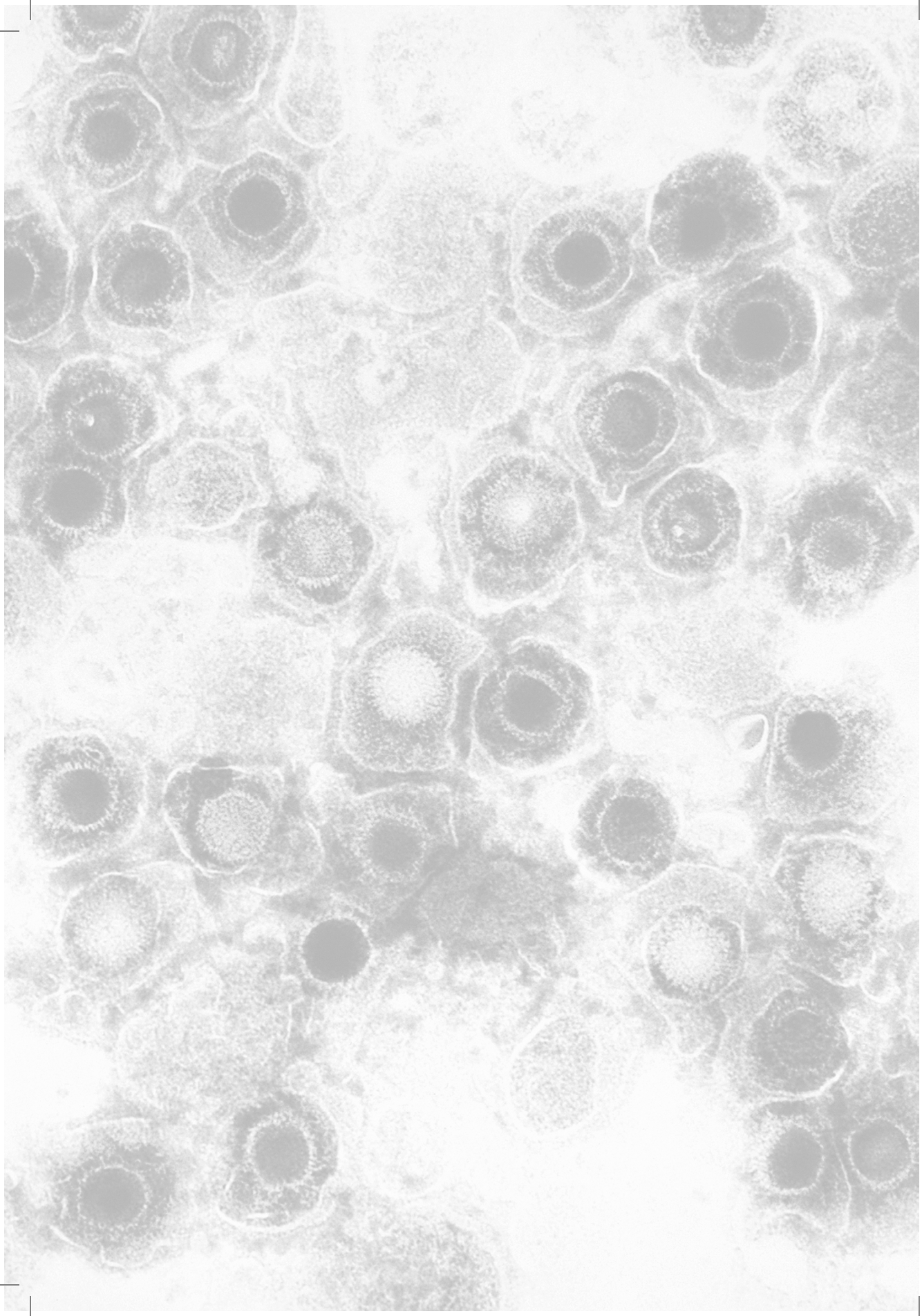
It always seems impossible until it's done
— Nelson Mandela



Curriculum Vitae

Curriculum Vitae

Julia Gunkel was born on the 12th of June 1985 in Ipoh, Malaysia. She had an international upbringing, growing up in Germany, China and Singapore. Her passion for medicine was ignited after an internship in 2003 under dr. Norbert Felgenhauer at the university hospital Klinikum rechts der Isar in Munich Germany. In 2004 she graduated from high school at the United World College of South East Asia in Singapore. Following a gap-year in Beijing, China she moved to Utrecht, The Netherlands to do a bachelor in biomedical sciences at the University College Utrecht in Utrecht. In 2008 she was admitted to the Selective Utrecht Medical Maser (SUMMA), a shorted (4 year) medicine program at University of Utrecht.

In 2011 she started her research project as part of her medical degree, on congenital and postnatal cytomegalovirus infections under the supervision of dr. Malgosia Maciolek-Verboon at the Neonatal Intensive Care Unit (NICU) of the Wilhelmina Children's Hospital in Utrecht, The Netherlands. This research project culminated in this thesis. In 2013, she finished her medical studies and started working full-time on her PhD. Concurrently, to she completed the second phase of the Effective Perinatal Care in Europe (EPICE) study in the Utrecht region under the supervision of dr. Arno van Heijst and dr. Corine Koopman-Esseboom. In 2015, she temporarily worked as a pediatric resident at the Medisch Centrum Haaglanden Westeinde Hospital in The Hague for nine months. From the end of 2015 until 2016 she completed her PhD thesis at the NICU at the Wilhelmina Children's Hospital in Utrecht.

Julia is married to Willem and they currently live in Singapore.

Notes