Postnatally acquired cytomegalovirus infections in preterm infants

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Postnatally acquired cytomegalovirus infections in preterm infants

Postnataal verworven cytomegalovirus infecties bij prematuur geboren kinderen

(met een samenvatting in het Nederlands)

Proefschrift

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" You don't need eyes to see, you need vision "

Maxi Jazz (Faithless) - Reverence

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List of abbreviations

AABR	Automated auditory brainstem response
ABR	Auditory brainstem response
AD	Axial diffusivity
AOIW	Age of onset of independent walking
AU	Arbitrary units
BPD	Bronchopulmonary dysplasia
BSITD-III	Bayley scales for infant and toddler development III
BW	Birth weight
CA	Corrected age
CI	Confidence interval
CID	Cytomegalic inclusion disease
CMV	Cytomegalovirus
CS	Caesarian section
CT	Computed tomography
cUS	Cranial ultrasonography
dB	Decibel
DBS	Dried blood spot
DNA	Deoxyribonucleic acid
DQ	Developmental quotient
DTI	Diffusion tensor imaging
EEG	Electroencephalogram
FA	Fractional anisotropy
GA	Gestational age
GLC	Germinolytic cyst
GMDS	Griffiths mental developmental scales
GMH	Germinal matrix haemorrhage
IgA	Immunoglobulin A
IgG	Immunoglobulin G

IgM	Immunoglobulin M
IVH	Intraventricular haemorrhage
LSV	Lenticulostriate vasculopathy
MD	Mean diffusivity
MRI	Magnetic resonance imaging
NA	Not available
NEC	Necrotizing enterocolitis
NICU	Neonatal intensive care unit
OR	Odds ratio
PCR	Polymerase chain reaction
PDA	Patent ductus arteriosus
PHVD	Posthaemorrhagic ventricular dilatation
PVE	Periventricular echogenicity
PVL	Periventricular leukomalacia
RD	Radial diffusivity
RDS	Respiratory distress syndrome
ROI	Region of interest
SGA	Small for gestational age
SD	Standard deviation
SNHL	Sensorineural hearing loss
TEA	Term-equivalent age
VLBW	Very low birth weight
WM	White matter

1 General introduction and outline of the thesis

Historical perspective

Professor of pathology Hugo Ribbert is considered to be the first to present the histopathology of cytomegalovirus (CMV) infection during a meeting of the Naturhistorischen vereins der Rheinlande und Westfalens in 1881.[1] In his presentation he described "large cells" in the kidney and parotid glands of an infant deceased of syphilis. Twenty-three years later Jesionek and Kiolemonoglou described "**owl eye cells**" in kidney, lung and liver in their paper on "protozoan like structures in the organs of an inherited infected luetic fetus",[2] and thereafter, Ribbert also described his findings.[3]

Goodpasture and Talbot proposed in 1921 to name the pathological findings **cytomegalia** (from Latin: cyto = cell, megalia = large) **infantum** because of the typically large aspect of these cells predominantly found in fetuses and infants.[4] Also, they noted that similar large cells could be seen in varicella zoster infected tissue and assumed that it was highly unlikely that these cytomegalic changes were caused by protozoa. In 1950, Wyatt et al. reviewed case studies of infants and adults with histopathologic changes associated with cytomegalia infantum and called the disease **cytomegalic inclusion disease** (CID), which is still a commonly used synonym to congenital CMV infection.[5] They assumed that the disease was caused by a virus. Subsequently, Fetterman managed in 1952, to diagnose CID through detection of infected cells in urine of congenitally infected infants.[6] Almost at the same time, small "virus-like particles" were reported by Minder in the halo of intranuclear inclusions by electron microscopy.[7] Then, in 1956, following the introduction of cell culture methods, three laboratories led by Smith, Rowe and Weller independently and almost simultaneously managed to isolate the virus that caused CID.[8–10] The laboratory of Smith had actually isolated the virus already in 1955. Unfortunately, as her laboratory previously isolated murine CMV,[11] her paper on human CMV was rejected based on the possibility of contamination. After isolation of the virus in a second patient her findings were acknowledged. The laboratories of Rowe and Weller used cell cultures to identify the causative agents of respiratory infections and toxoplasmosis, respectively, and incidentally isolated CMV. After exchanging the samples and confirming the results in their own laboratories, they concluded it was the same pathogen.[12] Finally, in 1960, Weller named the virus **cytomegalovirus**.[13]

Cytomegalovirus

Human CMV, also known as human herpesvirus 5 is the largest double stranded DNA-virus in the family of *Herpesviridae*. Its genome consists of approximately 235 kbp.[14] Together with the other mammalian CMVs and human herpesviruses 6 and 7 it belongs to the subfamily of *betaherpesvirinae*, which is characterized by its large genome size (over 200 open reading frames, which likely encode proteins) and relatively long replication cycle.[15] Their mechanism to establish latency and chronic shedding through asymptomatic intermittent reactivation of the virus ensures both lifelong persistence of CMV after primary infection as well as transmission to new hosts.[16] The complex mechanisms of CMV latency and reactivation are still poorly understood. During latency, episomes – circular formations of the viral genome – are maintained in the host cells in a quiescent state, without incorporation of viral DNA in the DNA of the cell.[17] After exposure to certain cytokines, the virus reactivates at restricted sites[18] and from there the virus is persistently shed.[16] Reinfection with a different CMV strain also may result in chronic shedding of the virus.[19] CMV can be shed through all body fluids, including blood, urine, liquor, breast milk, sputum, semen, vaginal excretions and amniotic fluid.

Newborns may acquire CMV *in utero* (congenital CMV infection), in the birth canal during vaginal delivery (perinatal CMV infection) or after birth (postnatal CMV infection).

Diagnostic methods

Congenital CMV infection is diagnosed when CMV is detected in body fluids within 2-3 weeks after birth, whereas CMV infection is perinatally or postnatally acquired when body fluids are found positive for CMV at a later age but had been negative within 3 weeks after birth.

In the past, a diagnosis of CMV infection was made by isolation of the virus from body fluids inoculated on cultured fibroblasts. In case of congenital infection this usually took a few days, but exclusion of CMV infection would take 3-4 weeks. In 1984, the introduction of a reliable culture method in which monoclonal antibodies were used to determine CMV infection considerably accelerated the diagnostic process.[20] Subsequently, new diagnostic tools were developed and new microbiological assays further improved the understanding and detection of CMV. Direct detection of CMV DNA using polymerase chain reaction (PCR) amplification was first introduced in 1988 by Demmler et al., offered great advantages over other diagnostic methods, and is

nowadays the gold standard for diagnosis of CMV infection.[21] The TaqMan based real-time PCR assay is a rapid, sensitive and specific further development of the original PCR for detection and quantification of CMV in different clinical samples such as urine, plasma and dried blood spots without the use of cell cultures.[22] Real-time PCR enables assessment of viral load, and to compare it to clinical presentation as well as efficacy of antiviral treatment.

In 1990, the genome of a laboratory strain of CMV, AD169, was first sequenced by Chee et al.[23] Subsequently, by sequencing individual CMV genes like UL144, the CMV genome was found to be quite variable resulting in the definition of various CMV genotypes.[24,25] It was hypothesized that CMV sequence variation, and thus different genotypes, is associated with differences in CMV disease manifestations and tissue tropism.[26,27] Furthermore, insight in and understanding of CMV variability may improve vaccine development and antiviral treatment, and may provide more insight into antiviral drug resistance.[28]

CMV infection induces the production of anti-CMV antibodies. The presence of anti-CMV IgM antibodies or low avidity anti-CMV IgG antibodies in serologic tests indicates a recent infection.[29] Nowadays, serology is not frequently used for the diagnosis of CMV infection in infants. However, if a patient's specimen collected shortly after birth is not available, CMV PCR in combination with CMV serology in dried blood spot cards may be used to determine congenital CMV infection in infants.[30,31]

Congenital CMV infection

During pregnancy the fetus may acquire CMV after maternal primary infection, reactivation or reinfection with a different CMV strain.[32] Birth prevalence of congenital CMV infection ranges between 0.4 – 2.0% worldwide.[33] In the Netherlands, the birth prevalence is estimated to be 0.5%.[34] The nature of maternal infection – primary infection versus reactivation or reinfection – is of great importance with respect to the risk of clinical manifestations and neurodevelopmental outcome of the infants. The highest risk of severe CMV disease and sequelae is found in newborns of mothers with primary infection.[35–37] Other viral or host factors (e.g. viral genotype, maternal antibodies, etc) that may contribute to congenital infection and the occurrence of disease symptoms at birth or later in life are largely unknown. Of all newborns with congenital CMV infection, approximately 10% is symptomatic at birth.[38,39] Clinical symptoms of congenital CMV disease at birth include intra-uterine growth retardation, microcephaly, hepatosplenomegaly, petechiae and purpura, icterus, chorioretinitis and thrombocytopenia.[38,40] Associated cerebral abnormalities as determined by neuro-imaging, include calcifications, ventriculomegaly, cysts, polymicrogyria, lissencephaly, hydrocephalus, periventricular leukomalacia and cerebellar hypoplasia.[41,42]

As CMV is a neurotropic virus, it is important to perform neuro-imaging in CMV-infected infants. The presence of cerebral abnormalities can be documented using cranial ultrasonography (cUS) and magnetic resonance imaging (MRI). In the past, computed tomography (CT) has been used to diagnose cerebral abnormalities in congenitally infected infants.[42,43] However, white matter (WM) changes and neuronal migration abnormalities caused by congenital CMV infection cannot be detected by CT. Furthermore, concerns were raised regarding the high radiation exposures.[44] The presence of gross structural WM abnormalities, e.g. cysts and calcifications, can be determined using cUS, which is safe, non-invasive and easily repeatable.[41,45,46] MRI, including diffusion weighted and diffusion tensor imaging is suitable to determine gross and microstructural WM changes, and migration abnormalities such as polymicrogyria and lissencephaly in infants with congenital CMV infection and provides more detailed information of the cerebellum, enabling us to diagnose associated cerebellar involvement.[41,47–49]

Approximately 20-30% of symptomatic infants die. Seventy to 80% of the surviving infants develops one or more sequelae, including sensorineural hearing loss (SNHL), cerebral palsy, epilepsy and neurodevelopmental delay.[38,39,50] An additional 10-15% of

infants without clinical symptoms of CMV disease at birth, may also develop sequelae. The majority of these will become manifest within the first two years of life.[51–53]

Previous studies have shown that high CMV load in blood and urine correlates with symptomatic CMV disease[54–57] and development of sequelae.[58] Differences between CMV genotypes may contribute to differences in virus-associated clinical manifestations.[59] UL55 or UL144 based genotyping has been applied to investigate either epidemiological or clinical associations of CMV infection.[24,25,60–64]

Although antiviral drugs against CMV are available (intravenous Ganciclovir® and oral Valganciclovir®), their efficacy in preventing or reducing sequelae in infants with congenital CMV infection is still uncertain. Consequently, there are no guidelines for treatment of these infants with antiviral medication. However, a reduction in SNHL has been documented in a small group of treated infants,[65] but improvement of neurodevelopmental outcome after antiviral treatment has not been confirmed sufficiently.[66]

To date, several trials with various anti-CMV vaccine candidates have been performed, but so far, none offered sufficient protection to congenital CMV infection.[67] Therefore, counselling and hygienic measures during pregnancy remain of great importance to prevent congenital CMV infection.[68]

Perinatal and postnatal CMV infection

Excretion of CMV in the genital tract of pregnant women at delivery has been previously reported and may cause CMV transmission during vaginal delivery.[69] Recently, however, it has been shown that CMV transmission during delivery is not as frequent as transmission through breast milk.[70–73]

Transmission through breast milk

Breast milk of CMV seropositive mothers is the most important source of CMV infection in newborn infants. The risk of acquiring a postnatal CMV infection through infected breast milk was first acknowledged in 1967 by Diosi et al.[74] Subsequently, Stagno et al.[75] demonstrated in 1980 that postnatal CMV infection in term infants was nearly always asymptomatic. In 2001, Hamprecht et al.[71] documented that 96% of CMV seropositive mothers of preterm infants shed CMV in breast milk, with a general onset at the first week post partum. Subsequently, CMV load increases until a maximum at 4-8 weeks after birth and then slowly decreases to undetectable levels at 9-12 weeks.[71,76–79] Low levels of CMV DNA in colostrum may be related to high levels of anti-CMV IgA and lactoferrin.[80,81]

CMV shedding in breast milk of seropositive mothers most likely occurs after local reactivation in the breast and rarely through systemic CMV reactivation, as maternal serum anti-CMV IgG and IgM levels remain stable during virolactia,[82] and CMV DNA is usually undetectable in sputum and urine.[76] Furthermore, CMV load in cell-free milk whey is significantly higher compared to the cellular fraction of breast milk.[71,82]

While 96% of seropositive mothers sheds the virus in breast milk,[71] the mother-to-infant CMV transmission rate is reported to range between 6-59%.[83] This broad range may reflect differences in study population and breast milk handling.[83,84] Furthermore, postnatal CMV transmission is likely to be influenced by the presence, levels and avidity of anti-CMV antibodies in mother and infant.[85,86] In several studies, high levels of maternal anti-CMV IgG in serum were reported as risk factor of CMV transmission.[69,87]

Epidemiology

Approximately 50% of pregnant Dutch mothers is CMV seropositive depending on geographical region and ethnicity,[88] which is similar to other western European countries like the United Kingdom (58%)[89] and Germany (52%).[70,71] CMV seropositivity is very high (97-99%) in non-native Dutch women (Moroccan, Turkish and Carribean ethnicity) compared to native Dutch women.[88] In many countries, CMV seropositivity among

pregnant women reaches 95-98% (Brazil, Italy, Mexico, China, Russia).[32,90] Differences in CMV seropositivity and breast milk handling between countries accounts for a broad range in incidence of postnatal CMV infection in preterm infants.[83,84] There are no studies on the epidemiology of postnatal CMV infection in the Dutch population.

Clinical manifestations, laboratory tests and neuro-imaging

Postnatal CMV infection in term-born infants is self-limiting and nearly always without any clinical symptoms.[75] However, preterm infants seem to be at risk of developing symptomatic CMV infection during hospitalization.[91] Clinical symptoms of postnatal CMV infection in preterm infants may include sepsis-like illness, pneumonia, hepatitis, cholestasis and necrotizing enterocolitis.[71,92,93] These clinical symptoms may be associated with thrombocytopenia, neutropenia and mild C-reactive protein elevation.[91] Neuberger et al.[73] have shown that postnatal CMV infection exacerbates pre-existing morbidity.

Low birth weight, early CMV transmission, early onset of DNAlactia and high viral load in milk whey were associated with a higher risk of symptomatic infection.[70,71] Maschmann et al.[70] studied 33 preterm infants <32 weeks of gestation, of whom 48% presented with clinical symptoms or laboratory signs of CMV disease.

In contrast to congenital infection, the association between CMV genotypes and clinical manifestations of postnatal CMV infection in preterm infants has not been studied previously.[56,60]

Similarly, cerebral imaging results of postnatally infected preterm infants have not been reported, with exception of our paper describing the development of lenticulostriate vasculopathy (LSV) as determined by cUS in two preterm infants with postnatal CMV infection.[25]

Long-term outcome

Data on long-term sequelae of postnatal CMV infection in preterm infants are scarce and limited to small study populations.[94] Even less is known about the effects of postnatal CMV infection on cerebral tissue or hearing in preterm infants. Since newborn premature infants may be comparable to the fetus in late gestation, one can imagine that postnatally infected preterm infants develop sequelae similar to those found in late trimester congenital infection. SNHL is a common complication of congenital CMV infection and may even develop after many months or years.[39,50] In the first study on long-term outcome by Paryani et al.[95] 17% of postnatally infected preterm infants had EEG abnormalities and 14% developed a severe handicap (presence of developmental

quotient [DQ] <70, severe neuromuscular impairment, profound SNHL, or profound loss of vision) at three years of age. However, there were no differences between infected and non-infected infants with respect to SNHL at 3 years of age.[95] In 2004, Vollmer et al.[72] reported follow-up results of 22 preterm infants with breast milk acquired CMV infection. The hearing of all infected infants was normal and their neurodevelopmental outcome was not different from a control group at 2 and 4.5 years of age.[72] However, neuro-imaging data of these patients were not described. Recently, Bevot et al.[96] have reported the neurodevelopmental outcome and hearing results of the cohort of Vollmer at school age. Motor and cognitive performance in both infected and non-infected infants were within the normal range.[96] However, a significant difference in motor and cognitive performance in infants with postnatal CMV infection compared to non-infected infants was reported. Hearing was normal in all infected infants. In the majority of other small case series, postnatally infected preterm infants had a normal neurodevelopmental outcome.[75,87,97,98] Still, sporadic case reports describe severe sequelae, such as SNHL,[99] and chorioretinitis.[100]

Prevention and treatment

To reduce the risk of postnatal CMV transmission in very low birth weight infants preventive measures are recommended in several countries.[101,102] Different methods, like pasteurization or freezing of breast milk have been studied to reduce the risk of postnatal infection. Freezing decreases CMV load in the breast milk, but does not sufficiently prevent infection.[87,103–105] However, CMV transmission rates were lower and protective factors of breast milk were preserved. Pasteurization procedures successfully inactivate CMV in breast milk, but also decrease beneficial properties of breast milk.[106,107]

Currently, there are no guidelines for treatment of preterm infants with postnatal CMV infection. In several severe symptomatic life-threatening cases the use of Ganciclovir®[108,109] or intravenous anti-CMV antibodies[110] was reported. However, since the great majority of postnatally infected preterm infants is asymptomatic, antiviral treatment can generally not be recommended.

Aims of the thesis

Postnatally acquired CMV in preterm infants is an important and frequent infection among NICU patients. Various preventive measures, including freezing or pasteurization of breast milk, are recommended in several countries to prevent CMV transmission from mother to infant through infected breast milk. After a few infants were diagnosed with postnatal CMV infection at our NICU in whom cerebral calcifications had developed, several questions arose with regard to the neurodevelopmental outcome and hearing of these infants. Also, neuro-imaging data in preterm infants were lacking in the scientific literature.

The aims of this thesis were:

1. To describe the epidemiology and risk factors of postnatal CMV infection in preterm infants.
2. To evaluate neuro-imaging, long-term neurodevelopmental outcome, and hearing of preterm infants with postnatal CMV infection.

Outline of the thesis

Chapter 1 presents a general introduction of the topic and the aims and outline of the thesis.

In **Chapter 2** we have described risk factors of a postnatally acquired CMV infection and cUS results in a large cohort of preterm infants.

In **Chapter 3** we have examined the risk of postnatal CMV infection with respect to serum anti-CMV IgG levels of mothers and their preterm infants at birth.

In **Chapter 4** we have studied the correlation between CMV load and disease severity. Urine CMV load of infants with congenital CMV infection was compared to urine CMV load of preterm infants with postnatal CMV infection

In **Chapter 5** we have studied the correlation between CMV genotypes and disease severity. CMV UL55 and UL144 genotype distribution was compared in congenitally and postnatally infected infants.

In **Chapter 6** we have examined cerebral white matter using an MRI technique (diffusion tensor imaging) and neurodevelopmental outcome at 16 months corrected age using the Griffiths Mental Developmental Scales in infants with postnatal CMV infection.

In **Chapter 7** we have studied the hearing of preterm infants with postnatal CMV infection using (automated) auditory brainstem response testing during the first and second year of life.

In **Chapter 8** we have examined the neurodevelopmental outcome of a cohort of preterm infants using the Bayley Scales of Infant and Toddler Development III (BSITD-III) to determine neurodevelopmental outcome of infants with postnatal CMV infection at 24 months corrected age.

Chapter 9 provides a summary, discussion and future directions of research.

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2 Postnatally acquired cytomegalovirus infection in preterm infants: a prospective study on risk factors and cranial ultrasound findings

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Abstract

Objective

To study risk factors and cranial ultrasound (cUS) findings in a large cohort of preterm infants, admitted to a neonatal intensive care unit and diagnosed with postnatally acquired cytomegalovirus (CMV) infection.

Study design

This prospective, observational study was performed from April 2007 until June 2009 among 315 infants born < 32 weeks of gestation. Postnatal CMV infection was diagnosed by CMV PCR in urine collected at term-equivalent age. In CMV positive infants, congenital infection was excluded. We compared the clinical and demographic data, feeding pattern, and cUS results of infected and non-infected patients. Logistic regression analysis was performed.

Results

In 39 of 315 infants the diagnosis of postnatal CMV infection has been made. The majority of CMV infected infants (33/39 [85%]) did not develop any symptoms of CMV infection. The most important, independent risk factors of postnatal CMV infection were non-native Dutch maternal origin (odds ratio [OR] 9.6 [95% confidence interval 4.3 -21.5]) and breast milk (OR 13.2 [95% CI 1.7 – 104.5]). The risk of infection significantly increased in infants with lower gestational age (OR 0.7 [95% CI 0.5-0.9]). Lenticulostriate vasculopathy (LSV) was significantly more often present in infants with CMV infection (OR 4.1 [95% CI 1.9 – 8.8]).

Conclusions

Postnatal CMV infection is an asymptomatic infection among preterm infants. Infants with lower gestational age are at greatest risk of postnatal CMV infection, especially when fed with fresh breast milk from their non-native Dutch mother. LSV not present at birth but confirmed at term-equivalent age can suggest a postnatal CMV infection.

Introduction

Infection with human cytomegalovirus (CMV) is the most common viral infection in neonates. Transmission of the virus from mother to child may occur during pregnancy (congenital CMV infection), during childbirth (perinatal CMV infection), or after birth (postnatal CMV infection). Congenital CMV infection occurs in 0.3 – 2.0% of all deliveries worldwide. About 10% of the infants who are symptomatic at birth may present with cerebral abnormalities such as polymicrogyria, lissencephaly, hydrocephalus, cerebellar hypoplasia, germinolytic cysts, subependymal pseudocysts and cerebral calcifications (including lenticulostriate vasculopathy (LSV)), with subsequent neurodevelopmental impairment and sensorineural hearing loss.[1] A further 10-15% of infants with asymptomatic infection at birth is estimated to develop sensorineural hearing loss early in life. Perinatal / postnatal CMV infection may be acquired during passage of the birth canal, transfusion of CMV-positive blood products, via direct contact with other body fluids (e.g. saliva) from CMV excreting parents and from breast milk of their CMV seropositive mothers.[2–7] Clinical signs and symptoms of postnatally acquired CMV infection include pneumonia,[6] enteritis,[8] cholestasis, hepatosplenomegaly, sepsis-like illness, thrombocytopenia, and neutropenia.[5,6,8] In one study, 42% of infected infants developed clinical or laboratory abnormalities (neutropenia and thrombocytopenia).[6] Based on sporadic case reports it is suggested that very low birth weight infants may be at increased risk of symptomatic infection.[4,9] Cranial ultrasound findings in preterm infants with postnatally acquired infection with this neurotropic virus have not yet been described.

The aim of this study was to assess risk factors of postnatally acquired CMV infection among preterm infants below 32 weeks of gestational age admitted to the neonatal intensive care unit. Furthermore, we evaluated the presence and evolution of cranial ultrasound abnormalities (calcifications including lenticulostriate vasculopathy, germinolytic cysts and subependymal pseudocysts) from birth until term-equivalent age.

Materials and methods

From April 2007 until June 2009 all preterm infants with GA below 32 weeks admitted to the NICU of a level 3 hospital, the Wilhelmina Children's Hospital, University Medical Center in Utrecht, the Netherlands were included. The study was approved by the Medical Ethical Committee.

Study design

Preterm infants admitted to our NICU were prospectively screened for CMV DNA in urine by performing CMV PCR at term-equivalent age, during the first follow-up visit at 40-42 weeks postmenstrual age. Postnatally acquired CMV infection was diagnosed by a positive CMV PCR. Congenitally acquired infection was excluded by negative CMV PCR on a urine sample collected within 1 week after birth and stored at -80° Celsius. Transfusion-associated CMV infection was prevented by the exclusive use of CMV seronegative blood-products.

Exclusion criteria were: congenital CMV infection, death before term-equivalent age, severe cerebral abnormalities at birth, loss to follow-up, no available urine sample at term-equivalent age. Infants in whom urine was not collected after birth and the diagnosis of congenital CMV infection was excluded by CMV PCR on dried blood spots (Guthrie card) were also excluded from the statistical analysis.

The following demographic and clinical data were collected: gestational age, birth weight, gender, maternal ethnicity, mode of delivery, Apgar scores at 1 and 5 min, feeding, hearing test in the early neonatal period, respiratory distress syndrome, hemodynamically significant persistent ductus arteriosus, bronchopulmonary dysplasia, sepsis, use of inotropic agents, blood transfusions and duration of admission to the NICU.

Respiratory distress syndrome was diagnosed by respiratory and radiologic criteria for respiratory distress syndrome and requirement of at least 1 dose of surfactant.[10] Hemodynamically significant persistent ductus arteriosus was defined as a ductus arteriosus requiring closure either by Indomethacin or by surgery. Bronchopulmonary dysplasia was defined as need for ≥ 30% oxygen and/or positive pressure ventilation at 36 weeks postmenstrual age.[11] Sepsis was considered when clinical signs and symptoms of infection were present, C-reactive protein (CRP) was increased and blood or cerebrospinal fluid culture was positive.[12] Clinical sepsis was defined as presence of clinical signs and symptoms of infection, increased CRP level, and negative blood

culture. Necrotizing enterocolitis was defined as presence of clinical symptoms of abdominal infection with pneumatosis intestinalis on an abdominal radiograph, according to the Bell criteria.[13]

Breast milk was given by nasogastric tube in all infants and was neither pasteurized nor frozen before use. All infants were observed for signs of CMV infection (sepsis-like illness, pneumonia, and cholestasis). Hemoglobin, white blood cell and platelets count were determined weekly as part of standard patient care until discharge.

Virology

The CMV PCR is an internally controlled real-time PCR, performed as previously described.[14] DNA was extracted from urine samples using the MagnaPure system and the Total Nucleic Acid isolation kit (Roche, Almere, The Netherlands). Amplification was performed on a Taqman platform (ABI Prism 7500 HT; Applied Biosystems, Foster City, CA, USA). Our real-time CMV PCR has a sensitivity limit of 250 copies/ml on urine specimens. In our study, urine was chosen as the preferred diagnostic specimen as this material appeared to be the most sensitive for diagnosis of (congenital) CMV infection.[15] Infants in whom the Guthrie card was used for exclusion of congenital CMV infection were not included in analysis.

Cranial ultrasound

Cranial ultrasound was performed at birth in all infants, as part of standard care, using an ATL UM-4 mechanical sector scanner (Philips, Medical Systems, Best, The Netherlands)

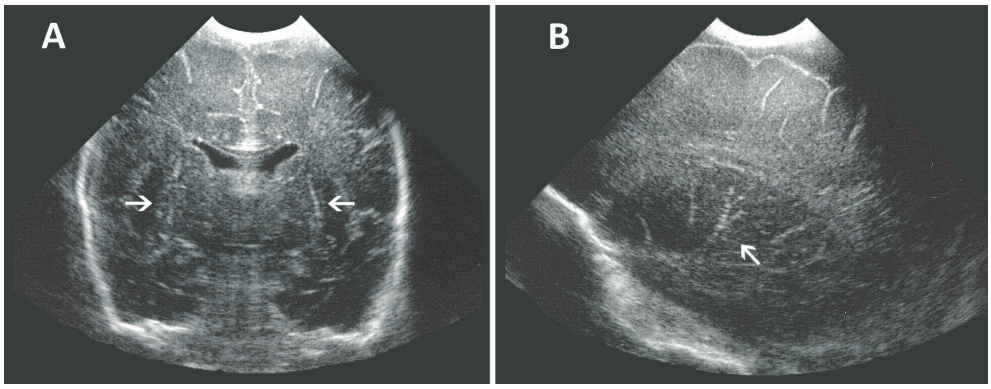

Figure 1. Coronal (A) and sagittal (B) cUS scans performed at term-equivalent age of a preterm infant born at 28 weeks of gestational age, showing bilateral lenticulostriate vasculopathy (arrows).

or an Aplio XG scanner (Toshiba Medical System, Zoetermeer, The Netherlands) with a multifrequency transducer (5 to 8.5 MHz) to ensure the best possible resolution. Cranial ultrasound was performed on a weekly basis until discharge and at the follow-up visit at term-equivalent age. The neonatologists performing cranial ultrasound were unaware of data on postnatally acquired CMV infection of the infants. Infants with severe cerebral abnormalities at birth were excluded from statistical analysis. Cranial ultrasound scans were evaluated uniformly for the presence of intraventricular haemorrhage, cysts (germinolytic cysts and subependymal pseudocysts), and calcifications such as linear or branching lenticulostriate vasculopathy (LSV) and minor (punctate) calcifications. Germinal matrix haemorrhage (GMH) and intraventricular haemorrhage (IVH) was graded according to Papile.[16]

Statistical analysis

Statistical analysis was performed using SPSS® (version 15.0, Lead Technologies, 2007). To determine risk factors associated with the development of CMV infection, clinical parameters were compared between preterm infants infected with CMV and non-infected infants by chi-squaretest for proportional variables, Fisher exact test, and 2-tailed unpaired student's T-test for continuous variables. Normality of data was tested by Kolmogorov-Smirnov test. Univariable logistic regression analysis was performed with acquired CMV (yes/no) as dependent variable and clinical parameters as independent variables, to calculate odds ratios and their 95% confidence intervals. Multivariable regression analysis was performed to investigate whether univariably statistically significant determinants relate independently to a postnatally acquired CMV infection. A p value < 0.05 was considered statistically significant.

Results

A total of 507 infants with GA below 32 weeks were admitted to our NICU during the study period. One hundred ninety two infants were excluded from analysis because of death before term-equivalent age (n=34), severe cerebral abnormalities at birth (n=12), congenital CMV infection (n=3), loss to follow-up (n=22) and because urine for CMV PCR was not collected at term-equivalent age (n=112) or after birth (n=9). In these 9 infants congenital CMV infection could not be excluded by performing CMV PCR in urine. There were no statistically significant differences between the eligible and non-eligible infants, in whom urine could not be collected, regarding gestational

age (median 29.7 wks and 30.4 wks, respectively) and ethnicity of the mother (65/315 [21%] and 35/192 [18%], respectively). Forty-eight twins and 3 triplets were included. Postnatal CMV infection was diagnosed in 39/315 (12%) preterm infants. Congenital infection in CMV positive infants at term-equivalent age was excluded by a negative result of CMV PCR in urine collected shortly after birth in all. Demographic and clinical characteristics of studied infants are shown in Table 1. Infants with postnatal CMV infection had a significantly lower gestational age and birth weight than non-infected

Table 1. Demographic and clinical characteristics of studied infant

Characteristic	CMV Positive n = 39	CMV negative n = 276	P
Demographic characteristics			
Gestational age, median, wk (range)	29.0 (25.0 – 31.0)	29.9 (24.9-31.9)	0.001
Birth weight, median, g (range)	1060 (660 - 1725)	1240 (600 – 2150)	0.008
Male gender, n (%)	24 (61)	153 (55)	0.472
Non-native Dutch maternal origin, n (%)	23 (59)	42 (15)	< 0.001
Vaginal delivery, n (%)	28 (72)	136 (49)	0.008
Breast milk, n (%)	38 (97)	215 (78)	0.004
Clinical characteristics			
Apgar 1 min, median (range)	8 (1 – 10)	7 (0 – 10)	0.585
Apgar 5 min, median (range)	9 (5 – 10)	9 (1 – 10)	0.974
RDS, n (%)	13 (33)	119 (43)	0.246
PDA, n (%)	9 (23)	54 (19)	0.608
Mechanical ventilation, n (%)	19 (48)	136 (49)	0.948
Sepsis, n (%)	10 (25.6)	86 (31)	0.483
BPD, n (%)	0 (0)	6 (2)	0.352
Use of inotropic agents, n (%)	10 (26)	97 (35)	0.241
Number of transfusions, median, n (range)	1 (0 – 8)	1 (0 – 18)	0.414
Abnormal ALGO hearing test, n (%)	0 (0)	4 (2)	0.453
NICU admission, median, days (range)	27 (4 – 98)	19 (3 – 138)	0.090

RDS - respiratory distress syndrome; PDA - persistent ductus arteriosus; BPD - bronchopulmonary dysplasia

infants. The majority of CMV infected infants 23/39 (59%) had a non-native Dutch mother (of Mediterranean, Caribbean, or Eastern European origin) compared to 42/276 (15%) of non-infected infants (p<0.001). Breast milk and vaginal delivery were also significantly different between infected and non-infected infants (p=0.004 and p=0.008, respectively). Subsequent multivariable regression analysis (Table 2) showed that non-native Dutch maternal origin (OR 9.3 [95% CI 4.1 – 20.9], p<0.001) and breast milk (OR 13.4 [95% CI 1.7 - 105.6], p=0.014) were the strongest independent risk factors of postnatally acquired CMV infection. While birth weight is related to gestational age multivariable regression analysis was also performed without birth weight. The risk of CMV infection significantly decreased for each additional week of gestational age (OR 0.7 [95% CI 0.5-0.9], p=0.001).

Cranial ultrasound

The results of cranial ultrasound in both groups of infants (CMV-infected and non-infected) are shown in Table 3. None of the infants developed subependymal pseudocysts. Germinolytic cysts were present in both groups at term-equivalent age. However, in contrast to CMV-infected infants, development of germinolytic cysts in non-infected infants was more often related to resolving GMH-IVH at term-equivalent age. The incidence of LSV increased fourfold in CMV infected infants at term-equivalent age compared to non-infected infants (OR 4.1 [95% CI 1.9 – 8.8], p < 0.001). It was found bilaterally in 6/13 (46%) and 11/30 (37%) of the infected and non-infected infants, respectively and right-sided in 5/13 (38%) and 10/30 (33%), respectively. Left-sided LSV was less common among infected infants 2/13 (15%) compared to 9/30 (30%) of non-infected infants. There were no significant differences between the subgroups. Development of LSV was not related to the presence of IVH after birth.

Morbidity among CMV positive infants

Eighty-five percent (33/39) of CMV positive infants did not have any clinical or laboratory signs of CMV infection during the postnatal period. These infants were diagnosed with CMV infection by screening at term-equivalent age. Six of 39 infants (15%) developed clinical signs of infection during admission to the NICU but only in one of them CMV PCR was performed at that time to prove CMV infection. In the remaining five infants the diagnosis of CMV infection could not be confirmed or excluded because CMV PCR was not performed.

Table 2: Univariable and multivariable regression (with and without birth weight) of significant characteristics

Characteristics	Univariable odds ratio (95% CI)	P	Multivariable odds ratio (95% CI)	P	Multivariable odds ratio without BW (95% CI)	P
Gestational age, wk	0.7 (0.6 - 0.9)	0.001	0.8 (0.5 - 1.1)	0.114	0.7 (0.5 - 0.9)	0.001
Birth weight, g	0.99 (0.9 - 1.0)	0.009	0.99 (0.9 - 1.0)	0.383	-	-
Non-native Dutch maternal origin	8.0 (3.9 - 16.4)	<0.001	9.3 (4.1 - 20.9)	<0.001	9.6 (4.3 - 21.5)	<0.001
Vaginal delivery	2.6 (1.3 - 5.5)	0.010	1.9 (0.8 - 4.8)	0.162	1.6 (0.7 - 3.7)	0.242
Breast milk	10.8 (1.5 - 80.1)	0.020	13.4 (1.7 - 105.6)	0.014	13.2 (1.7 - 104.5)	0.014

Table 3: Cranial ultrasound results in studied infants

Characteristic	CMV positive n = 39	CMV negative n = 276	P
GMH-IVH after birth, n (%)	14 (36)	73 (26)	0.217
Germinolytic cysts after birth, n (%)	1 (3)	14 (5)	0.491
Germinolytic cysts at TEA, n (%)	6 (15)	38 (14)	0.792
Germinolytic cysts at TEA not related to resolving GMH-IVH, n (%)	4 (10)	14 (5)	0.256
Minor calcifications after birth, n (%)	0 (0)	3 (1)	0.513
Minor calcifications at TEA, n (%)	5 (13)	31 (11)	0.770
LSV after birth, n (%)	0 (0)	1 (0)	0.707
LSV at TEA, n (%)	13 (33)	30 (11)	< 0.001

GMH – germinal matrix haemorrhage; IVH – intraventricular haemorrhage; LSV – lenticulostriate vasculopathy; TEA – term-equivalent age

Discussion

This prospective study reports on risk factors for postnatal CMV infection and neuro-imaging in a large cohort of preterm infants < 32 weeks GA. The risk of CMV infection increased significantly when the infant had a non-native Dutch mother, received breast milk and had a low gestational age. Development of cerebral calcifications, especially LSV was significantly more often identified in CMV infected infants compared to non-infected infants at term-equivalent age. To the best of our knowledge this is the first study which describes cranial ultrasound findings in preterm infants with postnatal CMV infection.

Previous studies have already shown that breast milk is the main source of postnatally acquired CMV infection in preterm infants.[17] It is also documented that 96% of CMV seropositive women have CMV reactivation with shedding of virus or the presence of CMV DNA in breast milk within several days after delivery.[5] Mother-to-child transmission rates of CMV in preterm infants range from 10 to 37% and depend on feeding practice.[4,5,7] In our cohort the risk of infection increased 13 times in infants who received breast milk. All breast-fed infants were given fresh untreated milk from their own mother. Breast milk is the most important source of nutrients for preterm infants and is, due to its advantages, widely used in spite of the risk of CMV transmission. A number of interventions has been proposed to inactivate CMV in breast milk prior to feeding. Pasteurization appears to be efficient in eliminating CMV, but concerns exist regarding the immunological and nutritional quality of the breast milk after intervention.[18] Freezing of breast milk at -20° C reduces, but does not completely prevent the risk of CMV transmission to a preterm infant.[19] Therefore this intervention has to be evaluated in clinical studies.

Ethnicity was a strong risk factor for postnatal CMV infection in our NICU. The risk of CMV infection was 9 times higher in infants born from non-native Dutch mothers compared to infants born from native Dutch mothers. Thirty-five percent (23/65) of infants from non-native Dutch mothers were infected, compared to 6% (16/250) of infants from native Dutch mothers. It would be interesting to be informed on seroprevalence of CMV among the mothers of all admitted preterm infants. However, data on CMV seroprevalence among ethnically diverse groups of pregnant women in the Netherlands have been reported previously.[20] According to this study the CMV seroprevalence among pregnant women from Mediterranean (Morocco, Turkey) or Caribbean (Surinam and The Netherlands Antilles) origin was 85-100% compared to

40-55% among native Dutch women.

Our study shows that the risk of CMV infection decreases significantly (30%) with each week of increasing gestational age. Since birth weight is related to gestational age, multivariable regression analysis was also performed without birth weight. In this analysis gestational age appeared to be an independent risk factor for postnatally acquired CMV infection. We presume that the absence of prenatally acquired anti-CMV antibodies before the 28th week of gestational age in combination with the presence of CMV in breast milk is the most plausible explanation for the increased risk among infants with lower gestational age. Other factors such as CMV load and duration of breast feeding may play an additional role in the transmission.[4]

It is of interest that we did not find an association with high morbidity among the postnatally CMV-infected preterm infants as previously reported.[6,9,20] On the contrary, in our study the majority (85%) of infected infants did not develop any signs of CMV infection. Moreover, in contrast to other studies, symptomatic CMV infection was not associated with bronchopulmonary dysplasia, necrotizing enterocolitis or prolonged hospital stay.[2,3,21]

Our study shows that development of LSV at term-equivalent age, seen on cranial ultrasound, occurred significantly more often in infants with postnatal CMV infection compared to non-infected infants, 33% (13/39) and 11% (30/276), (OR 4.1 [95% CI 1.9 – 8.8], $p < 0.001$) and was not related to the presence of GMH-IVH after birth.[22] Development of LSV, several weeks after birth, in preterm infants has been reported, but the association with postnatal CMV infection has not been described previously. In addition, cerebral calcifications (including LSV), germinolytic cysts, and subependymal pseudocysts are common neuro-imaging abnormalities observed in symptomatic infants with congenital CMV infection.[23,24] The brain of the preterm infant is known to be very susceptible to viral infections. Therefore, LSV may be the result of necrotizing inflammation of lenticulostriate arteries during CMV infection in preterm infants. A contributing role of CMV in vascular pathology has been reported previously.[25] It is of interest that a prospective study on the incidence and evolution of LSV in a cohort of preterm infants showed that LSV developed more often in infants with lower gestational age.[26] As we showed that the risk of CMV infection increases with lower gestational age, it is possible that this observation could be partly explained by postnatal CMV infection. Although the presence of germinolytic cysts was not statistically different at term-equivalent age in both groups of patients, in contrast to non-infected infants, the development of germinolytic cysts in most CMV infected infants was not associated

with a resolving germinal matrix haemorrhage. The relationship of LSV and germinolytic cysts with postnatally acquired CMV infection and neurodevelopmental outcome still has to be determined in our population. The development of cranial abnormalities is of interest in view of the neurological development of these preterm infants including hearing and learning disabilities. Recently, the relationship between LSV and development of sensorineural hearing loss has been described in a small group of infants with congenital CMV infection.[27] All infants included in our study will be seen at the follow-up clinic until at least five years of age.

A limitation of this study is a high rate (22%) of missing inclusions at term-equivalent age due to difficulties with urine collection in young infants in the follow-up clinic. However, the analysis of demographic and clinical data of excluded infants did not show any differences between excluded and included infants.

In conclusion, the most important, independent risk factors of postnatally acquired CMV infection among preterm infants are non-native Dutch maternal origin, breast milk and low gestational age. The majority of infected infants does not develop any clinical signs or symptoms of CMV infection. Since one-third of infected infants developed LSV in the postnatal period, assessment of long-term outcome is recommended.

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3 Maternal and neonatal anti-cytomegalovirus IgG level and risk of postnatal cytomegalovirus transmission in preterm infants

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Abstract

Immunological mechanisms influencing the risk of mother-to-child cytomegalovirus (CMV) transmission in preterm infants have not been studied sufficiently. In this study, the correlation between maternal and neonatal serum anti-CMV IgG levels and risk of postnatal CMV transmission in preterm infants was assessed. Anti-CMV IgG levels of 79 CMV seropositive mothers and their 94 infants were determined in peripheral blood samples collected within 3 days after delivery. Postnatal CMV infection was detected in 39/94 (41%) infants by PCR on urine at term-equivalent age (gestational age 40 weeks) after congenital infection was excluded. Maternal or infant anti-CMV IgG levels were not significantly different between infants with and without postnatal CMV infection. The anti-CMV IgG infant-mother ratio showed a significant positive correlation with gestational age (range 25-32wks, R^2=0.218, p<0.001), reaching 1.0 at 32 weeks of gestation. Anti-CMV IgG infant-mother ratio was significantly lower in infants with postnatal CMV infection (p=0.015). In conclusion, the risk of postnatal CMV transmission is related to low gestational age and low anti-CMV IgG infant-mother ratio.

Introduction

Postnatal cytomegalovirus (CMV) infection is common in preterm infants. In very-low-birth-weight (VLBW) infants it may result in short-term morbidity, including pneumonitis and sepsis-like illness with thrombocytopenia.[1] However, the great majority of infants with postnatal CMV infection does not develop any signs and symptoms of CMV disease.[2,3] Since CMV negative blood products are used in the treatment of VLBW infants, breast milk is assumed to be the main source of postnatal CMV infection among preterm infants. Although 96% of CMV seropositive mothers sheds the virus in breast milk, only 10-50% of preterm infants fed with CMV-infected breast milk acquires postnatal infection.[4] Similar to other viral infections CMV-specific antibody titers in mother and infant may influence the acquisition of CMV infection during the postnatal period.[5,6] A high maternal anti-CMV IgG level has been suggested previously as a risk factor for increased transmission of CMV in preterm infants.[7–9]

In this study, the correlation between maternal and neonatal anti-CMV IgG levels of preterm infants with and without infection was assessed. Furthermore, the degree of maternal to infant antibody transfer with respect to gestational age and risk of CMV transmission was investigated.

Materials and methods

Study subjects

From April 2007 until December 2010 CMV seropositive mothers and their preterm infants, born before 32 weeks of gestation and admitted to the Neonatal Intensive Care Unit of the University Medical Center, Utrecht, the Netherlands were included. Serum remaining of a venous blood sample drawn from the mothers at delivery (5 ml) and from their preterm infants at admission (0.5 ml) for reason of blood group compatibility testing, was frozen at -20°C and used for anti-CMV IgG analysis. The following demographic and clinical data of the mothers were collected: maternal age at delivery, (non-)Dutch origin, gravidity, parity and mode of delivery. The following data of the infants were collected: gestational age, birth weight, enteral feeding, presence of lenticulostriate vasculopathy on cranial ultrasonography performed at term-equivalent age (40 weeks of gestation), respiratory distress syndrome, mechanical ventilation, bronchopulmonary dysplasia, persistent ductus arteriosus, sepsis and number of blood-product transfusions. The Internal Review Board of the University Medical Center

Utrecht, The Netherlands approved this study.

Postnatal CMV infection

Urine of all infants was collected twice: within two weeks after birth and at term-equivalent age. Urine collected shortly after birth was frozen at -80°C. Urine collected at term-equivalent age was examined for the presence of CMV by quantitative CMV PCR, as described previously.[3] Infants with a positive CMV PCR in urine collected at term-equivalent age were considered infected postnatally if CMV PCR in urine collected shortly after birth was negative. When this early urine sample was not available, CMV PCR and anti-CMV IgM analysis was performed on dried blood spots to exclude congenital CMV infection, as described previously.[10–12] The real-time CMV PCR has a sensitivity limit of 50 copies/ml on plasma samples, of 250 copies/ml on urine specimens and of approximately 1000 copies/ml on dried blood spots (DBS), which was confirmed by participation in an external quality assessment program.[13] CMV infection was considered symptomatic when signs and symptoms of postnatal CMV infection were present (sepsis-like illness, respiratory disease, thrombocytopenia), bacterial blood culture was negative and CMV PCR of urine and plasma was positive.

Anti-CMV IgG enzyme immune-assay

Venous blood samples of the mothers were used to determine anti-CMV IgG using an enzyme immune-assay, VIDAS® (BioMerieux, Boxtel, The Netherlands), performed as recommended by the manufacturer. When the mother was anti-CMV IgG seropositive, anti-CMV IgG was determined in the blood sample of her infant, irrespective of postnatal CMV infection. Results are expressed as arbitrary units (AU) per ml. The threshold of seropositivity was 4 AU/ml anti-CMV IgG. Anti-CMV IgG infant-mother ratios, a measure of maternal to infant antibody transfer, were calculated and correlated to gestational age. In addition, the relation between these ratios and transmission risk for the total group and three subgroups (≤27 weeks of gestation, 28-29 weeks and ≥30 weeks) was calculated.

Statistical analysis

Statistical analysis was performed using PASW Statistics® (version 18.0.0, SPSS, Inc, Chicago USA, 2009). Figures were produced using GraphPad Prism® (version 5.03, GraphPad Software, Inc, La Jolla USA, 2009).

For the purpose of calculating means, antibody titers were transformed into $\log_{10}$ values. Anti-CMV IgG level of the infant whose CMV antibodies were undetectable was assigned

a value of 1 AU/ml. After testing for normality of data by the Kolmogorov-Smirnov test, demographic and clinical data, anti-CMV IgG level and ratio of CMV positive and CMV negative infants and their mothers was compared using Chi-squaretest for proportional variables and two-tailed non-parametric Mann-Whitney U test for continuous variables. Correlations were determined using the Spearman rank coefficient. A p-value <0.05 was considered statistically significant.

Results

Study subjects

Mothers

From April 2007 until December 2010, 184 mothers were eligible for anti-CMV IgG analysis of whom 102 (55%) mothers were anti-CMV IgG seropositive. In 30 infants of 23 seropositive mothers, serum was not available for analysis. Therefore, data of 79 CMV seropositive mothers and their 94 infants were eligible for analysis (Table 1).

Infants

Of the 94 infants examined, 39 (41%) infants acquired postnatal CMV infection in the neonatal period as determined by positive CMV PCR in urine at term-equivalent age (Table 1). Congenital infection was excluded in all infants, in 35/39 (90%) of them using CMV PCR in urine after birth and in four infants by negative CMV PCR on DBS cards. Only one infant (3%) presented with symptoms (isolated thrombocytopenia) of postnatal CMV infection. The remaining 55/94 (59%) infants did not acquire postnatal CMV infection. Fifteen non-identical twins were included, of whom five (33%) were CMV positive. In one (7%) twin, one infant acquired postnatal CMV infection and the other infant did not. The infant with postnatal CMV infection was born by vaginal delivery, while the infant without infection was born by caesarean section. Other risk factors of postnatal CMV infection (breast milk and anti-CMV IgG levels of 25 AU/ml) were equal in both infants. For the analyses, each twin was considered individually.

CMV infection occurred less frequently in infants born by caesarean section than in infants born through vaginal delivery (11/39 [28%] versus 28/39 [72%], p<0.001, respectively). Gestational age was significantly lower in CMV positive infants compared to CMV negative infants (mean 28.3wk [SD 1.6] and 29.5wk [SD 1.7], p=0.001, respectively). Lenticulostriate vasculopathy which developed weeks after birth,

occurred more often in CMV positive infants compared to CMV negative infants (15/39 [39%] and 8/55 [15%], p=0.008, respectively).

Table 1. Demographic and clinical characteristics of study subjects.

	Mothers of CMV positive infants n = 33	Mothers of CMV negative infants n = 46	P
Age at birth, mean, yr (SD)	29.5 (4.6)	31.7 (5.9)	0.077
Non-Dutch origin, n (%)	18 (55)	20 (44)	0.332
Caesarean section, n (%)	10 (30)	32 (70)	0.001
Gravida, mean, n (SD)	1.8 (0.9)	2.2 (1.3)	0.115
Para, mean, n (SD)	0.6 (0.8)	0.8 (1.0)	0.315
	CMV positive infants n = 39	**CMV negative infants n = 55**	**P**
Gestational age, mean, wk (SD)	28.3 (1.6)	29.5 (1.7)	0.001
Gestational age ≤27wk, n (%)	14 (36)	9 (16)	0.030
Birth weight, mean, g (SD)	1171 (282)	1235 (325)	0.319
Caesarean section, n (%)	11 (28)	37 (67)	< 0.001
Breast milk, n (%)	39 (100)	55 (100)	1.000
Lenticulostriate vasculopathy at term-equivalent age, n (%)	15 (39)	8 (15)	0.008
Respiratory distress syndrome, n (%)	14 (36)	25 (46)	0.354
Mechanical ventilation, n (%)	15 (39)	17 (31)	0.446
Bronchopulmonary dysplasia, n (%)	2 (5)	1 (2)	0.368
Persistent ductus arteriosus, n (%)	11 (28)	12 (22)	0.478
Sepsis, n (%)	10 (26)	18 (33)	0.459
Transfusions, mean, n (SD)	2.3 (3.1)	2.0 (3.0)	0.679

Anti-CMV IgG analysis

There was no significant difference between maternal anti-CMV IgG levels of CMV positive infants and CMV negative infants at birth with respect to gestational age (Figure 1A-D).

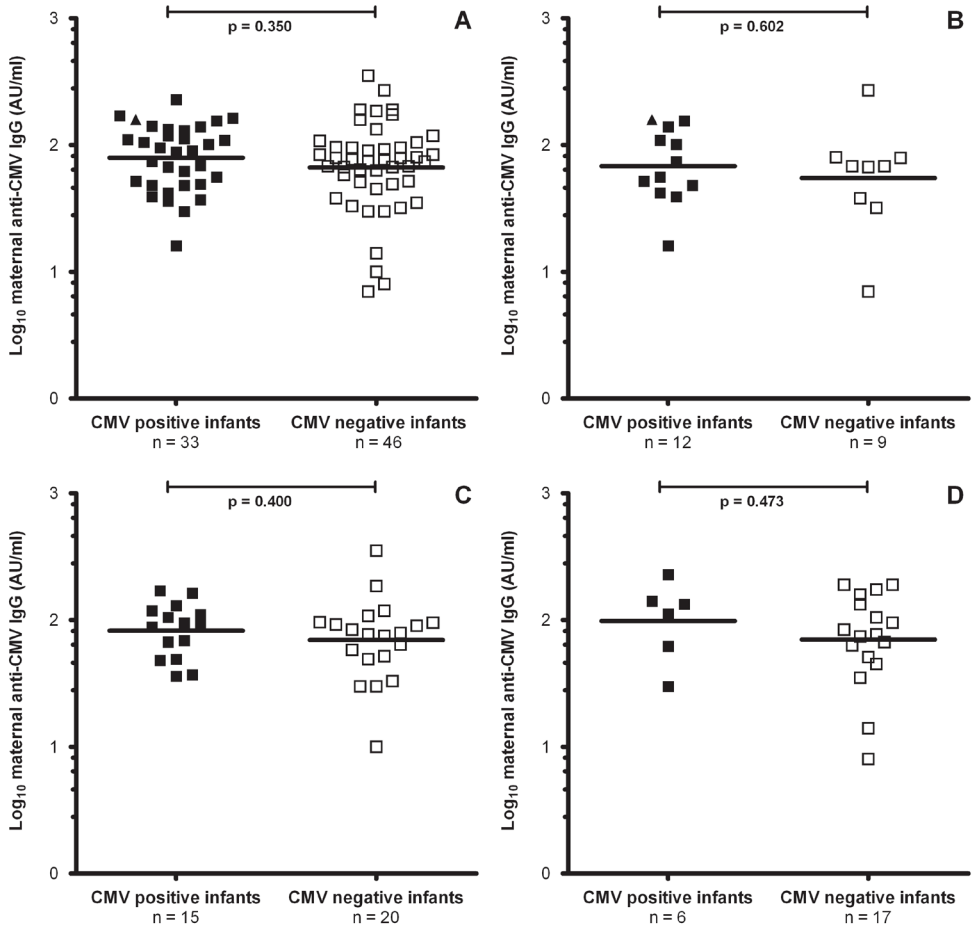

Figure 1. Log_{10} anti-CMV IgG levels in mothers of CMV positive and CMV negative infants (A) and in gestational age subgroups ≤27 weeks (B), 28-29 weeks (C) and ≥30 weeks (D).

Bars in scatter dot plot represent geometric mean anti-CMV IgG (AU/ml). Filled squares represent mothers of infants with asymptomatic postnatal CMV infection, the filled triangle the mother of the infant with symptomatic postnatal CMV infection and open squares mothers of infants without postnatal CMV infection.

The anti-CMV IgG levels of CMV positive infants compared to CMV negative infants were not different in the total group and in 3 different gestational age subgroups (Figure 2A-D).

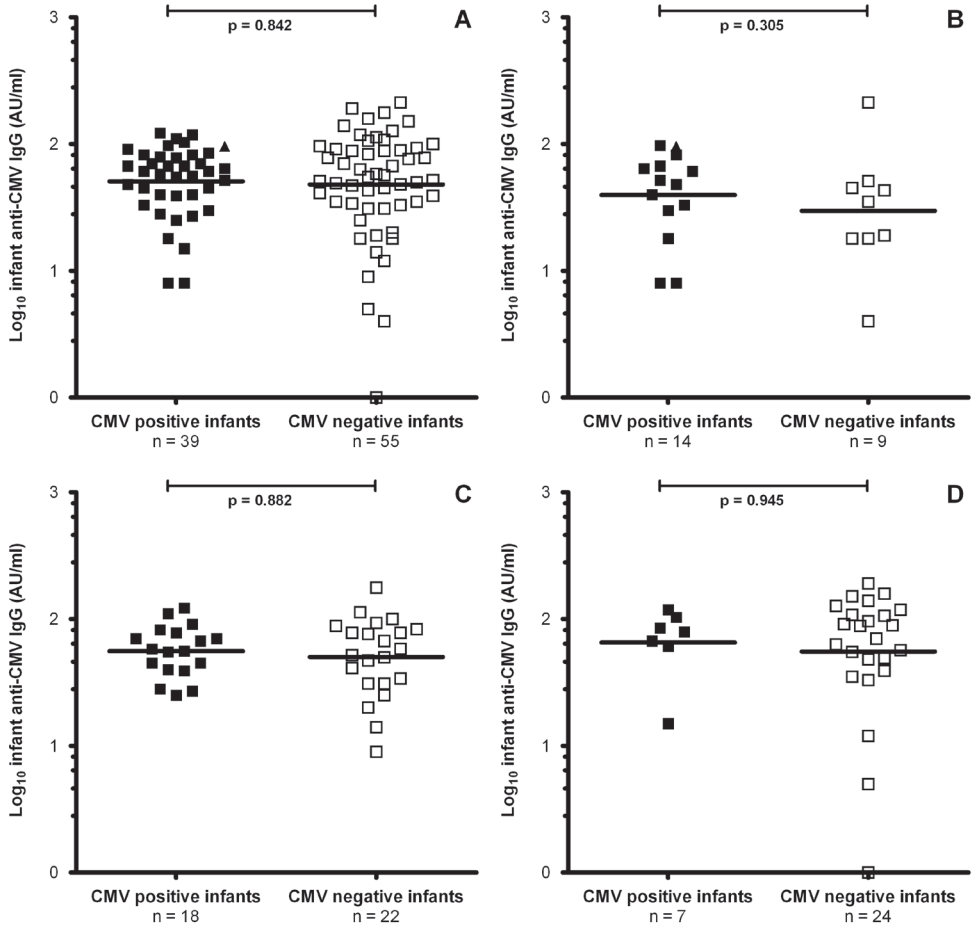

Figure 2. Log_{10} infant anti-CMV IgG levels in CMV positive and CMV negative infants (A) and in gestational age subgroups ≤27 weeks (B), 28-29 weeks (C) and ≥30 weeks (D).

Bars in scatter dot plot represent geometric mean anti-CMV IgG (AU/ml). Filled squares represent infants with asymptomatic postnatal CMV infection, the filled triangle the infant with symptomatic postnatal CMV infection and open squares infants without postnatal CMV infection.

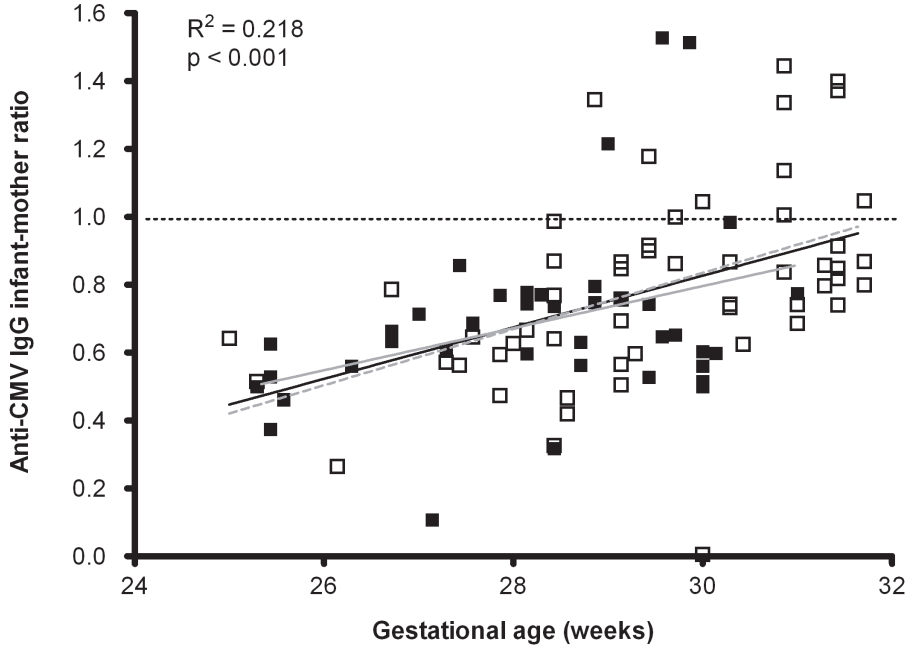

Figure 3. Anti-CMV IgG infant-mother ratio with respect to gestational age

A regression line of all infants is depicted in solid black, of the infants with postnatal CMV infection in solid gray and of the infants without infection in dotted gray. R^2- and p-value represent Spearman Rank correlation coefficient of all infants. Filled squares represent infants with postnatal CMV infection and open squares infants without postnatal CMV infection.

Spearman rank correlation coefficient calculation showed a significant, linear correlation between the degree of maternal-infant antibody transfer (anti-CMV IgG infant-mother ratio) and gestational age (range 25-32wks, R^2=0.218, p<0.001) reaching 1.0 at 32 weeks of gestation (Figure 3). Anti-CMV IgG infant-mother ratio was significantly lower in infants with postnatal CMV infection compared to infants without infection (Figure 4A). A significantly lower ratio was related to risk of CMV infection in the subgroup born from 30 weeks of gestation (p=0.007) (Figure 4D). Analysis of maternal and infant anti-CMV IgG levels between infants who were born through vaginal delivery or caesarian section did not yield significant differences (data not shown). Postnatal development of lenticulostriate vasculopathy was not associated with anti-CMV IgG levels of mother and infant or infant-mother ratio (data not shown).

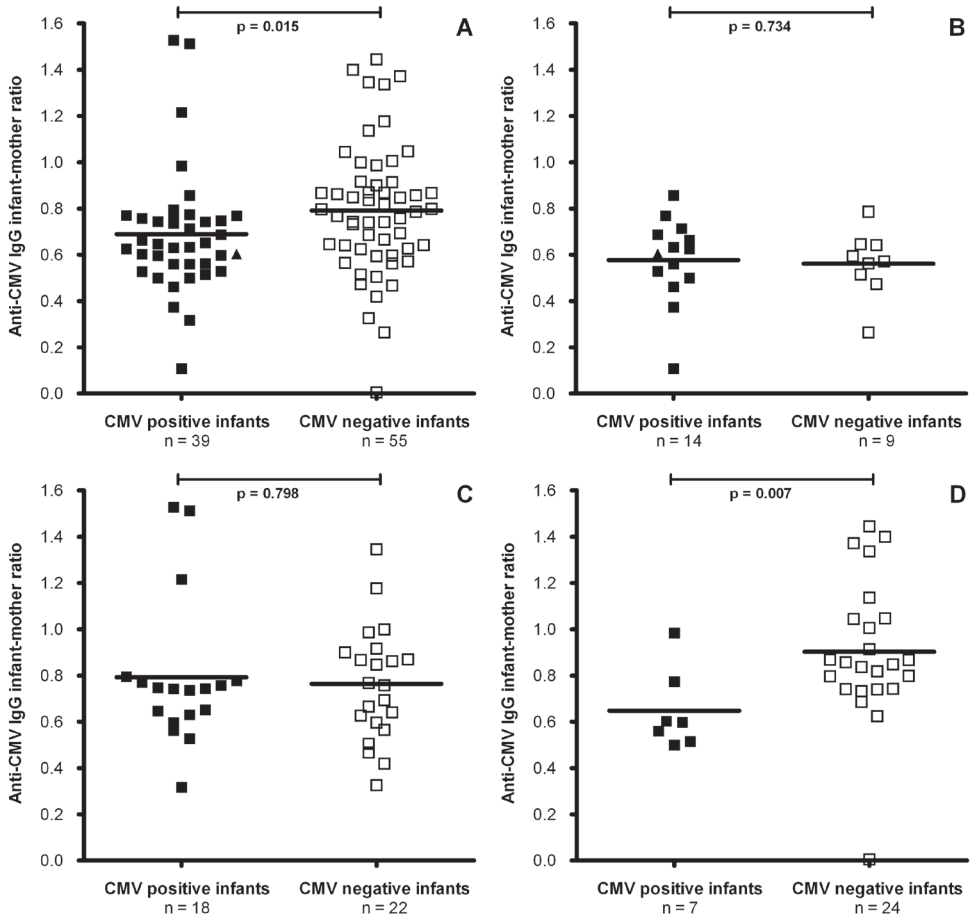

Figure 4. Anti-CMV IgG infant-mother ratio in CMV positive infants and CMV negative infants (A) and categorized in three groups depending on gestational age ≤27 weeks (B), 28-29 weeks (C) ≥30 weeks (D).

Bars in scatter dot plot (A, B, C & D) represent mean anti-CMV IgG infant-mother ratio. Filled squares represent infants with asymptomatic postnatal CMV infection, filled triangle the infant with symptomatic postnatal CMV infection and open squares infants without postnatal CMV infection.

Discussion

This study shows that the risk of postnatal mother-to-child CMV transmission in preterm infants is not related to absolute maternal or infant anti-CMV IgG levels. In addition, the absolute anti-CMV IgG levels at birth were not different in infants with and without postnatal CMV infection, born ≤27 weeks of gestation who are known to be at highest risk of postnatal CMV infection.[3,14]

Maternal and neonatal anti-CMV IgG levels and risk of CMV transmission have been studied sporadically and only in limited numbers of patients. In addition to high CMV load and prolonged shedding of viable virus in the breast milk of seropositive mothers, high serum maternal anti-CMV IgG was suggested as a marker for CMV reactivation in the mother and therefore, as an important risk factor of increased CMV transmission.[7–9,15] However, in accordance with a recent study by Ehlinger et al.[6] in 30 CMV seropositive mothers, present study did not confirm these findings. This study shows that it is not possible to identify infants at risk for postnatal CMV infection using absolute levels of maternal or infant anti-CMV antibodies.

As antibody titers in preterm infants and their mothers were not related to increased risk of CMV transmission, it was hypothesized that this risk may be related to anti-CMV IgG infant-mother ratio, which is indicative of the degree of maternal-fetal antibody transfer. Transplacental transport of anti-CMV antibodies has not been studied previously. This is the first study to report a positive linear correlation between gestational age (range 25 weeks to 32 weeks) and anti-CMV IgG infant-mother ratio. Similar to data from studies on varicella zoster virus, the anti-CMV IgG infant-mother ratio reached 1.0 at 32 weeks of gestation.[16]

It is of interest that the anti-CMV IgG infant-mother ratio was significantly lower in infants with postnatal CMV infection compared to infants without infection. In a previous study, it is reported that lower gestational age is an independent risk factor of postnatal CMV infection in preterm infants.[3] The anti-CMV IgG infant-mother ratio, as documented in the present study, also depends on gestational age suggesting that infants with low gestational age may be at risk of CMV infection. In addition, in the infants with gestational age ≥30 weeks anti-CMV IgG infant-mother ratio was significantly lower in the infants with postnatal CMV infection. It may be that in these infants either transplacental transport of anti-CMV antibodies from mother to infant was limited, or the mothers had increased anti-CMV antibody titers because of recent CMV reactivation or reinfection.

Much of the mechanism of CMV reactivation in the mother and CMV shedding in breast milk is still unknown. However, excretion of the virus in colostrum and the genital tract after delivery suggests increased viral replication following CMV reactivation or re-infection during pregnancy.[9] In immunocompromised patients reactivation of CMV leads to an increase of anti-CMV specific antibodies and in healthy seropositive persons higher levels reflect asymptomatic reactivation.[17,18] Since anti-CMV antibody levels are related to reactivation, vulnerable infants with low anti-CMV IgG infant-mother ratio in whom the mothers may have high titers due to reactivation may be at risk of CMV infection.

Studying CMV transmission in twins may help to understand the immune responses after CMV infection. One pair of twins in this study was of particular interest, since both infants were seropositive with identical anti-CMV IgG levels at birth. However, only one of them acquired postnatal CMV infection. The mode of delivery, or differences in exposure to infected breast milk may account for this difference. Furthermore, differences in individual genetic susceptibility for CMV may also be responsible, similar to congenital cases reported previously.[19]

It is of note that only one infant with postnatal CMV infection presented with symptomatic disease. Therefore, the relation between anti-CMV IgG levels, anti-CMV IgG infant-mother ratio and symptomatic disease could not be evaluated.

As shown in a previous study, lenticulostriate vasculopathy determined at term-equivalent age, occurred more often in CMV positive infants compared to CMV negative infants.[3] However, this finding was not associated with differences in anti-CMV antibody titers or anti-CMV IgG infant-mother ratios.

A limitation of this study is that CMV load in breast milk was not determined, to support the assumption that postnatal infection is acquired through breast milk of CMV seropositive mothers. Since CMV infection occurred significantly more often in infants who were born through vaginal delivery and vaginal swabs were not taken, this route of transmission cannot be excluded. The infected genital tract is a well-known source of CMV transmission at delivery.[20] However, all infants examined received fresh breast milk from their own mother and breast milk is found to be the most important source of CMV for preterm infants. Ninety-six percent of CMV seropositive mothers of VLBW infants sheds the virus in breast milk for weeks with peak levels around one month after delivery,[4] while vaginal swabs are positive in 40% of the women at delivery.[9] Analysis of maternal and infant anti-CMV IgG levels between infants who were born through vaginal delivery or caesarian section did not yield significant differences.

In conclusion, absolute mother and infant anti-CMV IgG levels are not related to the risk of postnatal CMV infection in preterm infants. The risk of postnatal CMV transmission seems to be related to low gestational age and low anti-CMV IgG infant-mother ratio.

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4 Urine viral load and correlation with disease severity in infants with congenital or postnatal cytomegalovirus infection

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Abstract

Background

A correlation between cytomegalovirus (CMV) load in urine and severity of disease in congenitally infected infants has previously been reported. CMV load in postnatally infected infants has not been studied before.

Objective

To investigate CMV load in urine of infants with postnatal or congenital infection and correlate this with clinical symptoms of CMV disease and cerebral abnormalities.

Study Design

Infants admitted to our NICU between July 2000 and February 2010, and diagnosed with congenital or postnatal CMV infection were included. Clinical symptoms of CMV infection, cranial ultrasonography (cUS) and magnetic resonance imaging (MRI) findings were evaluated. CMV urine loads of postnatally infected infants were analyzed and compared with CMV urine loads of congenitally infected infants.

Results

Seventeen infants with congenital CMV infection and 45 infants with postnatal CMV infection were included. Thirteen/17 (76%) congenitally infected infants had clinical symptoms of CMV infection at birth and 11/17 (65%) had cerebral abnormalities diagnosed by neuro-imaging. None of the four asymptomatic infants had cerebral abnormalities. Of the postnatally infected infants 43/45 (96%) did not develop any clinical symptoms of CMV infection, but in 23/45 (51%) cerebral abnormalities such as lenticulostriate vasculopathy and germinolytic cysts were identified. The median CMV load in postnatally infected infants was significantly lower than in congenitally infected infants (1.0 x 10^5 copies/ml versus 8.5 x 10^6 copies/ml, $p<0.001$, respectively).

Conclusions

CMV load in urine is significantly lower in infants with postnatal CMV infection than in infants with congenital CMV infection irrespective of clinical symptoms of CMV infection or cerebral abnormalities.

Background

Cytomegalovirus (CMV) is the most common cause of congenital infection in newborns with a reported incidence of 0.2-2%.[1,2] These infants may be at risk of developing neurodevelopmental sequelae, including sensorineural hearing loss (SNHL). Previous studies have shown that high CMV load in blood and urine correlate with symptomatic CMV disease[3–6] and development of adverse sequelae.[7] In contrast to congenital CMV infection, acquisition of CMV in the postnatal period, mostly through breast milk of CMV-seropositive mothers, has been previously found to be a less significant health problem for newborns.[8] However, more recent studies described small numbers of preterm infants with postnatal infection who developed severe short-term and long-term morbidity.[9–12] Data on long-term developmental outcome and hearing in these preterm infants are limited and inconclusive.[13,14]

Objectives

We aimed to study the relationship between urine viral load in preterm infants with postnatal CMV infection and severity of disease, based on clinical symptoms of CMV disease and cerebral involvement determined by cranial ultrasonography (cUS) and magnetic resonance imaging (MRI). Furthermore, we aimed to compare the CMV urine load of these infants with the CMV urine load of congenitally infected infants.

Study design

Congenital CMV infection

From July 2000 until February 2010 all infants diagnosed with congenital CMV infection and admitted to the level 3 neonatal intensive care unit (NICU) of the University Medical Center Utrecht, The Netherlands, were included. Congenital CMV infection was confirmed by positive CMV PCR of urine collected within 2 weeks after birth. Symptomatic congenital CMV infection was diagnosed using CMV PCR of urine when signs and symptoms of CMV infection were present at birth (microcephaly, growth retardation, hepatosplenomegaly, hepatitis, petechiae, thrombocytopenia, chorioretinitis, or SNHL). In preterm infants with congenital CMV infection, CMV PCR of urine was repeated at term-equivalent age (TEA, 40 weeks gestational age) and the resulting viral load was used for analysis.

Postnatal CMV infection

Preterm infants (<32 weeks gestational age) admitted to our NICU from April 2003 until February 2010 and diagnosed with symptomatic or asymptomatic postnatal CMV infection during admission were included. From April 2003 until March 2007 CMV PCR of urine was performed in preterm infants who developed cerebral abnormalities suggestive of CMV infection as described previously.[15] From April 2007 until February 2010 all preterm infants were screened on presence of CMV in urine by PCR during a visit to the follow-up clinic at TEA. Congenital CMV infection was excluded in these infants by negative CMV PCR of urine collected within 2 weeks after birth. Symptomatic postnatal CMV infection was considered when the preterm infant presented with sepsis-like illness, thrombocytopenia and/or respiratory disease weeks after birth, negative bacterial blood culture, and positive CMV PCR of urine and plasma.

Quantitative Real-Time PCR

CMV PCR is an internally controlled real-time PCR, performed as previously described.[16] DNA was extracted from urine samples using the MagnaPure system and the Total Nucleic Acid isolation kit (Roche, The Netherlands). Amplification was performed on a Taqman platform (ABI Prism 7500HT; Applied Biosystems, USA). Our real-time CMV PCR has a sensitivity limit of 50 copies/ml in plasma samples, 250 copies/ml in urine specimens and approximately 1,000 copies/ml in dried blood spots, as described previously.[17,18]

Neuro-imaging

cUS was performed in all studied infants on admission to the NICU. In congenitally infected infants with cerebral abnormalities on the first cUS, cerebral MRI was performed. The presence of polymicrogyria, lissencephaly, hydrocephalus, cerebellar hypoplasia, germinolytic cysts, pseudocysts, and lenticulostriate vasculopathy (LSV) was evaluated in these infants. In preterm infants with postnatal CMV infection, cUS was repeated on a weekly basis until discharge. At TEA, the presence of calcifications in basal ganglia such as LSV and presence of germinolytic cysts was evaluated as described previously.[15] In the majority of the postnatally infected infants a cerebral MRI was performed at TEA, using a 3-Tesla Intera system or a 1.5-Tesla system (Philips, The Netherlands). MRI included sagittal T1, axial spin-echo T2, inversion recovery and diffusion-weighted imaging.

Statistical analysis

Statistical analysis was performed in PASW Statistics® (version 18.0, SPSS inc., 2009). Clinical symptoms of CMV disease, hearing and neuro-imaging results were analyzed for congenitally and postnatally infected infants using chi-squaretest and Fisher exact test. To compare the viral load between various groups, a non-parametric Mann-Whitney U test was performed. A p value < 0.05 was considered statistically significant.

Results

Congenital CMV infection

Seventeen (eleven term and six preterm) infants were diagnosed with congenital CMV infection (Table 1). Urine was collected shortly after birth in all infants (median age 1d, range 0-12d). Thirteen of 17 (76%) infants were symptomatic: growth retardation

Table 1. Characteristics of all studied infants

Characteristics	Congenital CMV infection n = 17	Postnatal CMV infection n = 62
Demographic and clinical characteristics		
Gestational age, median, wk (range)	38.0 (28.9 – 42.0)	28.9 (25.0 – 31.3)
Birth weight, median, g (range)	2775 (810 – 4600)	1055 (600 – 1725)
Male gender, n (%)	9 (53)	33 (53)
CMV viral load, mean, copies/ml (SD) *	39.2×10^6 (105.1×10^6)	2.3×10^6 (5.9×10^6)
Abnormal hearing test, n (%)	4 (24)	0 (0)
Mortality, n (%)	3 (18)	0 (0)
Clinical symptoms of CMV infection, n (%) **	13 (76)	3 (5)
Cerebral imaging		
LSV on cUS, n (%)	6 (35)	22 (35)
GLC on cUS, n (%)	6 (35)	8 (13)
MRI abnormalities, n (%) ***	5 (29)	3 (5)

CMV – cytomegalovirus; LSV – lenticulostriate vasculopathy; cUS – cranial ultrasonography, GLC – germinolytic cysts; MRI – magnetic resonance imaging

* p < 0.001; ** growth retardation, microcephaly, hepatitis, hepatosplenomegaly, petechia, thrombocytopenia, neutropenia and/or seizures; *** polymicrogyria, lissencephaly, holo-pros-encephaly, hydrocephalus, cerebellar hypoplasia, germinolytic cysts, pseudocysts

(2/17), microcephaly (1/17), hepatitis (2/17), hepatosplenomegaly (4/17), petechia (4/17), thrombocytopenia (8/17), neutropenia (1/17), seizures (1/17) and abnormal hearing test at birth (4/17). Eleven of 17 infants (65%) had cerebral abnormalities seen on MRI and/or cUS, including hydrocephalus (2/11), holoprosencephaly (1/11), lissencephaly (1/11), polymicrogyria (1/11), LSV (6/11) and germinolytic cysts (6/11). Two symptomatic infants and the 4/17 asymptomatic infants did not have cerebral abnormalities. In the four asymptomatic infants CMV PCR was performed because of proven CMV seroconversion during pregnancy in three and symptomatic CMV disease in the other part of a twin in one.

Postnatal CMV infection

Sixty-two infants (all preterm) were diagnosed with postnatal CMV infection at TEA. Congenital CMV infection was excluded in 45/62 (73%) infants by performing a CMV PCR in urine collected within 2 weeks after birth. The data from the remaining 17 infants in whom CMV PCR of dried blood spots was used, were excluded from the analysis (Table 1). Two/45 (4%) infants developed clinical signs of CMV infection; pneumonia (n=1) and sepsis-like illness with thrombocytopenia (n=1). In 23/45 (51%) infants, cerebral abnormalities (LSV in 17/23 and germinolytic cysts in 6/23) were found at TEA using cUS. In addition cerebral MRI was performed, in 22/45 infants. Whereas LSV can only be seen on cUS, the presence of germinolytic cysts detected by cUS was confirmed by MRI (table 1). Other cerebral abnormalities associated with CMV infection were not found. Hearing was tested in 44/45 (98%) infants at the age of 6-10 months (median 212 days) and was normal in all.

Virology

The median urine CMV load in infants with postnatally acquired CMV infection was significantly lower ($p<0.001$) compared to congenitally infected infants: 1.0 x 10^5 copies/ml versus 8.5 x 10^6 copies/ml, respectively (Figure 1). The median CMV load in infants with cerebral abnormalities was higher compared to infants without cerebral abnormalities in both congenitally infants (1.1 x 10^7 copies/ml versus 1.6 x 10^6 copies/ml, $p=0.088$, respectively) (figure 2A) and postnatally infected infants (1.7 x 10^5 copies/ml versus 8.6 x 10^4 copies/ml, $p=0.532$, respectively) (Figure 2B), but did not reach significance in both groups.

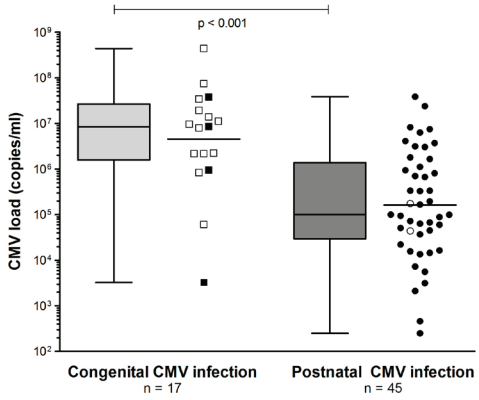

Figure 1. CMV load in congenitally and postnatally infected infants

Upper and lower borders of box plots represent the 25th and 75th percentile, bars represent median viral loads and whiskers represent minimum to maximum values. Bars in scatter dot plot represent mean viral loads after $\log_{10}$ transformation. Open symbols represent symptomatic disease and filled symbols represent asymptomatic disease.

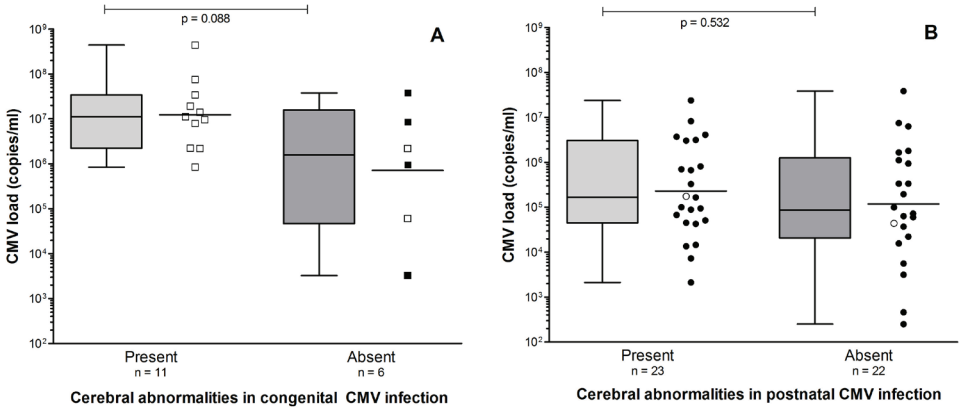

Figure 2. CMV load in infants with congenital (A) and postnatal (B) CMV infection in respect of cerebral abnormalities

Upper and lower borders of box plots represent the 25th and 75th percentile, bars represent median viral loads and whiskers represent minimum to maximum values. Bars in scatter dot plots represent mean viral loads after $\log_{10}$ transformation. Open symbols represent symptomatic disease and filled symbols represent asymptomatic disease. Cerebral abnormalities are: polymicrogyria, lissencephaly, hydrocephalus, cerebellar hypoplasia, germinolytic cysts, pseudocysts and lenticulostriate vasculopathy.

Discussion

Our data are the first showing that CMV load in urine of infants with postnatally acquired CMV infection is significantly lower than CMV load in urine of infants with congenital CMV infection.

Postnatally acquired CMV infection is common among preterm infants who receive breast milk from CMV seropositive mothers.[10,15] Short-term consequences of postnatal CMV infection in these preterm infants have been described previously.[9–11] Long-term consequences of this infection with regard to hearing and neurodevelopmental outcome seem to be favorable, but have not been studied sufficiently.[14,19,20] Nevertheless, in many countries interventions such as freezing or pasteurization of breast milk, are recommended to prevent CMV infection in preterm infants.[21]

Whereas 76% of congenitally infected infants in our study presented with clinical symptoms of CMV disease at birth, which is known to be associated with an increased risk of SNHL,[22] only two postnatally infected, preterm infants (4%) showed clinical symptoms compatible with CMV infection. In 43 infants the diagnosis of postnatal CMV infection was determined only by urine screening at TEA and the viral load was low. The association between low viral load and low risk of SNHL in congenital infected infants has been documented previously.[4] This is in line with the findings from our study, since SNHL was not detected in infants with postnatal CMV infection and a low viral load, at least until the follow-up visit at the age of 6-10 months.

Identifying central nervous system involvement in infants with CMV infection is important in view of their neurodevelopmental prognosis and hearing. Recently, we have found that LSV develops significantly more often in preterm infants with postnatally acquired CMV infection compared to non-infected infants suggesting central nervous system involvement.[15] However, the importance of solitary LSV and germinolytic cysts as markers of the development of SNHL in infants with congenital CMV infection is controversial.[23,24] Interestingly, the presence of cerebral abnormalities in postnatally infected infants was not related to a high viral load in our study, which may suggest that the risk of hearing loss and adverse outcome is low.

In contrast to infants with postnatal CMV infection, neuro-imaging showed severe cerebral abnormalities in the majority of infants with congenital CMV infection. Since we were not able to document a significant difference between the viral load of congenitally infected infants with cerebral abnormalities detected by neuro-imaging compared to the viral load of the few infants without cerebral abnormalities, more

attention should be paid to investigate this relationship and its prognostic relevance for long-term outcome in congenital CMV infection.

Central nervous system involvement is a serious condition in infants with congenital CMV infection in whom antiviral treatment should be considered.[25] However, the benefits of antiviral treatment in infants with asymptomatic infection have not been sufficiently established. Since infants with low viral load may have a lower risk of SNHL, highly toxic antiviral treatment should not be recommended in postnatally infected infants and asymptomatic congenitally infected infants without cerebral abnormalities until more data have been collected in favor of this treatment. Future clinical studies are required to elucidate whether preterm infants with postnatal CMV infection and low viral load are at risk of developing long-term sequelae.

A limitation of this study is the small number of infants without symptoms of congenital CMV infection (n=4). Therefore we were not able to observe differences in viral load between symptomatic and asymptomatic infants with congenital CMV infection, as previously reported.[4] The viral load in two infants with symptomatic postnatal CMV infection was low.

In conclusion, the results of our study show that CMV load in urine of preterm infants with postnatal CMV infection of whom the great majority do not develop any clinical signs of CMV infection, is significantly lower than the viral load of infants with symptomatic congenital CMV infection. Long-term neurodevelopmental outcome and hearing of infants with postnatal CMV infection and low CMV load will be assessed in the near future.

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5 Cytomegalovirus genotype distribution among congenitally and postnatally infected infants and the relation with disease severity

Submitted

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Abstract

Background

Congenital cytomegalovirus (CMV) infection is a leading cause of long-term sequelae. CMV is also frequently transmitted postnatally to preterm infants, but these infections are mostly asymptomatic. A correlation between CMV genotypes and clinical manifestations has been reported previously in infants with congenital infection, but not in preterm infants with postnatal infection. The aim of this study was to investigate CMV genotype distribution in congenital and postnatal CMV infection and its association with disease severity.

Methods

Infants admitted to the NICU between 2003-2010 and diagnosed with postnatal or congenital CMV infection were included. Classification of CMV isolates in genotypes was performed upon amplification and sequencing of the UL55 and UL144 genes of CMV. Clinical data, cerebral abnormalities, outcome and viral loads were studied in relation to genotype distribution.

Results

Genotyping results were obtained from 58 preterm infants with postnatal CMV infection and 13 infants with congenital CMV infection. Infants with congenital CMV infection were significantly more severely affected than infants with postnatal infection. Seventy-seven percent of these infants were symptomatic at birth, 2/13 died and 3/13 developed long-term sequelae. Postnatal disease was mild in all preterm infants and all had favourable outcome. The distribution of CMV genotypes was comparable for congenital and postnatal infection. UL55 genotype 1 and UL144 genotype 3 were predominant genotypes in both groups.

Conclusions

Distribution of UL55 and UL144 genotypes was similar in severe congenital and asymptomatic postnatal CMV infection suggesting that timing of the infection rather than CMV genotype is responsible for development of severe disease.

Introduction

Cytomegalovirus (CMV) infection is the most common congenital infection. Symptoms of CMV infection at birth include growth restriction, microcephaly, petechiae, jaundice, hepatomegaly, retinitis, hearing loss, cerebral abnormalities and thrombocytopenia. Congenital CMV infection may lead to death or neurologic sequelae, including sensorineural hearing loss in later life.[1,2]

CMV is also frequently transmitted postnatally to preterm infants admitted to the neonatal intensive care unit, mostly via breast milk of their CMV positive mothers.[3] While symptoms of postnatal CMV infection in preterm infants may include sepsis-like illness, pneumonia and thrombocytopenia, the great majority of these infants is asymptomatic.[4] Limited data suggest that neurodevelopmental outcome and hearing are favourable in these preterm infants.[5–7]

It has been hypothesized that differences between CMV strains may contribute to differences in virus-associated clinical manifestations.[8] In various studies, considerable genetic variability was found in CMV UL55 and UL144, enabling the assignment of specific CMV-genotypes for each of both genes. UL55 or UL144 based genotyping has been applied to investigate either epidemiological or clinical associations of CMV infection.[9–15] Correlations between CMV genotypes and clinical manifestations are still limited and controversial, mainly because of the heterogeneous composition of the investigated cohorts. The CMV genotype distribution among postnatally infected preterm infants has not been reported previously.

The aim of the current study was to determine whether congenital and postnatal CMV infections in preterm infants were caused by specific CMV UL55 and UL144 genotypes and to assess whether these genotypes were associated with CMV disease severity. In addition, the correlation between genotype distribution and viral load was evaluated.

Materials and methods

Study population

Newborn infants admitted to the neonatal intensive care unit of the University Medical Center, Utrecht, The Netherlands, and diagnosed with congenital or postnatal CMV infection between 2003-2010 were included. Congenital CMV infection was diagnosed if a CMV PCR in urine was positive within 2 weeks after birth.

Postnatal CMV infection in preterm infants was determined when the CMV PCR in urine collected at term-equivalent age was positive, while the urine collected within two weeks after birth was CMV negative. In infants of whom urine was not collected after birth, a negative CMV PCR on dried blood spots cards obtained within 3 days after birth was used to exclude congenital CMV infection, as previously described.[16]

Symptomatic postnatal CMV disease was diagnosed in infants with clinical manifestations of CMV disease during their admission (sepsis-like illness, pneumonia, thrombocytopenia), negative bacterial blood cultures and positive CMV PCR in urine and blood at time of symptoms.

Demographic and clinical data and CMV urine loads were collected. Cerebral abnormalities were evaluated using cranial ultrasonography and magnetic resonance imaging (MRI) during admission. Cranial ultrasonography was repeated on a weekly basis in preterm infants during their stay in the neonatal intensive care unit. The MRI was performed in term infants with congenital CMV infection shortly after birth and in preterm infants with congenital or postnatal CMV infection at term-equivalent age using a 3-Tesla Intera system or a 1.5-Tesla system (Philips, The Netherlands). The neurodevelopmental outcome was assessed by Griffiths Mental Developmental Scales test or Bayley Scales of Infants and Toddler Development III test from 12 months of age in all infants. Brainstem auditory evoked potentials were performed from 6 months of age to determine sensorineural hearing loss.

DNA extraction, Polymerase Chain Reaction and sequencing

Viral DNA was isolated from 100 µl urine using the Nuclisens MiniMAG (BioMérieux, Boxtel, The Netherlands) and eluted in 100 µl elution buffer. The purified DNA was subsequently used for amplification of the UL55 and UL144 genes, using primers described in Table 1, which are essentially based on Lurain et al.[9] and Stranska et al.[10] (Life Technologies, Foster City, CA, USA). A 616-bp fragment containing the UL144 coding sequence and a 532-bp fragment containing the UL55 coding sequence were

Table 1. Sequences of oligonucleotide primers used for CMV PCR and sequencing[12,27]

Primer name	Sequence
Primers used for amplification	
UL55_Outer_Forward	5`-TCCGAAGCCGAAGACTCGTA-3`
UL55_Outer_Reverse	5`-CATTCCTCAGTGCGGTGGTT-3`
UL55_Inner_Forward	5`-CTGCCAAAATGACTGCAACT-3`
UL55_Inner_Reverse	5`-ACATCACCCATGAAACGCGC-3`
UL144_Outer_Forward	5`-ACAAACCGCGGAGAGGATGA-3`
UL144_Outer_Reverse	5`-TCAGACACGGTTCCGTAAAG-3`
UL144_Inner_Forward	5`-GTTCGGCCCCATGAGTTATT-3'
UL144_Inner_Reverse	5`-GTGTGACTTCATCGTACCGT-3'
Primers used for sequencing	
UL55_Sequencing_Forward	5`-CTGCCAAAATGACTGCAACT-3`
UL55_Sequencing_Reverse	5`-ACATCACCCATGAAACGCGC-3`
UL144_Sequencing_Forward	5`-GTTCGGCCCCATGAGTTATT-3`
UL144_Sequencing_Reverse	5`-GTGTGACTTCATCGTACCGT-3`

amplified using the Expand-high fidelity amplification kit (Roche Applied Science, Penzberg, Germany). PCR was performed in a GeneAmp2700 thermal cycler (Life technologies), according to the following conditions: 1 hold at 94°C for 2 minutes, 10 cycles at 94°C for 15 seconds, 55°C for 30 seconds and 72°C for 1 minute, 20 cycles at 94°C for 15 seconds, 55°C for 30 seconds and 72°C for 1 minute and 5 seconds (increased by 5 seconds every cycle), final extension at 72°C for 7 minutes. Nested RT-PCR was performed according to the following conditions: 1 hold at 94°C for 2 minutes, 10 cycles at 94°C for 15 seconds, 55°C for 30 seconds and 72°C for 45 seconds, 10 cycles at 94°C for 15 seconds, 55°C for 30 seconds and 72°C for 50 seconds (increased by 5 seconds every cycle), final extension at 72°C for 7 minutes.

PCR-products were visualized upon agarose gel electrophoresis, purified using QIAquick PCR purification columns (Qiagen Gmbh, Germany) following the manufacturer's instructions and eluted in 30-100 µl nuclease-free water.

The purified PCR-products were subsequently used for cycle sequencing in a GeneAmp2700 thermal cycler, according to the following protocol: 25 cycles at 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes.

Reaction products were subsequently purified using EDTA-ethanol precipitation, prior to analysis on an ABI3700 automated sequencer (Life technologies), using the primers described in Table 1.

The obtained electropherograms were analyzed and edited using SeqScape® data analysis software, v2.6 (Life technologies). Full double stranded coverage was achieved for both amplified gene fragments. The obtained consensus sequences for each of the samples were then aligned with MEGA Alignment Explorer® and genotype classification was achieved with MEGA Tree Explorer® (MEGA, The Biodesign Institute, AZ, USA). UL144 sequences were classified into genotype 1A, 1B, 1C, 2 or 3, according to Lurain et al.[9], and UL55 sequences were classified into genotype 1, 2, 3, 4 or 5, based on several studies.[9,11,15,17–20] The reference sequences used for classification were obtained from GenBank, Pubmed.

Statistical analysis

Statistical analysis was performed in PASW statistics® (version 18.0, SPSS Inc., 2009). Proportional variables were analyzed using chi-square test or Fisher's exact test. Continuous variables were analyzed using a non-parametric Mann-Whitney U test. To compare CMV load between congenitally and postnatally infected infants logarithmic transformation was performed. Variances of CMV load between genotypes were analyzed with the non-parametric Kruskal-Wallis test. A p-value <0.05 was considered statistically significant.

Results

Clinical data

Clinical data of both groups of patients are summarized in Table 2. Infants with congenital CMV infection were more severely affected than postnatally infected infants. Ten (77%) presented at birth with symptoms of CMV disease: IUGR (n=5),microcephaly (n=2), hepatosplenomegaly (n=4), petechiae (n=3), jaundice (n=1), seizures (n=1), thrombocytopenia (n=6), anaemia (n=2), neutropenia (n=1). Cranial ultrasonography was performed in all infants and showed ventricular dilatation in two infants and presence of lenticulostriate vasculopathy in six (46%) and germinolytic cysts in seven (54%) infants. In addition, cerebral MRI was performed in seven symptomatic infants

Table 2. Demographic and clinical characteristics of 13 congenitally and 58 postnatally infected infants

	Congenital CMV infection n = 13	Postnatal CMV infection n = 58	P
Gestational age, mean, wk (SD)	36.5 (4.2)	28.4 (1.9)	< 0.001
Birth weight, mean, g (SD)	2530 (1030)	1140 (330)	< 0.001
Male gender, n (%)	8 (62)	33 (57)	0.759
Non-Dutch maternal origin, n (%)	3 (23)	27 (47)	0.121
Symptomatic CMV infection, n (%) [a]	10 (77)	5 (9)	< 0.001
Mortality, n (%)	2 (15)	0 (0)	0.002
Abnormal outcome / hearing, n (%)	3 (23)	0 (0)	< 0.001
Urine Log_{10} CMV load, median, copies/mL (IQR)	6.34 (1.62)	5.24 (1.43)	0.002
Severe MRI abnormalities, n (%) [b]	6 (86)	0 (0)	< 0.001
Calcifications on cranial ultrasonography at term-equivalent age, n (%)	6 (46)	20 (35)	0.429

a. Symptoms of congenital CMV infection included IUGR (n=5), microcephaly (n=2), hepatosplenomegaly (n=4), petechiae (n=3), jaundice (n=1), seizures (n=1), trombocytopenia (n=6), anemia (n=2), and neutropenia (n=1). Symptoms of postnatal CMV infection included pneumonia (n=3), and sepsis-like illness with thrombocytopenia (n=2)

b. MRI was performed in 7 congenitally infected infants and 30 postnatally infected infants. Severe MRI abnormalities included polymicrogyria (n=1), occipital cysts (n=1), ventricular dilatation (n=1) and abnormal white matter signal intensity (n=4)

and showed severe abnormalities in six infants, including polymicrogyria (n=1), occipital cysts (n=1), ventricular dilatation (n=1) and abnormal white matter signal intensity (n=4). Two infants died shortly after birth and three developed neurodevelopmental delay, epilepsy or sensorineural hearing loss at 8, 6 and 2 years, respectively. Three asymptomatic infants with congenital infection were born from mothers with proven primary CMV infection during late pregnancy, and had normal outcome.

Of 58 postnatally infected preterm infants, five (9%) developed symptoms of CMV disease (pneumonia [n=3], sepsis-like illness with thrombocytopenia [n=2]) while the others (91%) were asymptomatic and identified by screening only.

In contrast to infants with congenital infection, none of the infants with postnatal infection developed severe cerebral abnormalities (p<0.001). The presence of LSV and germinolytic cysts was seen on cranial ultrasonography in 20 (35%) and 8 (14%) infants, respectively, and was not associated with unfavourable neurodevelopmental outcome. No additional cerebral abnormalities were found using MRI, which was

Table 3. UL55 and UL144 genotype distribution of congenital and postnatal CMV infection.

	Congenital CMV infection n = 13	Postnatal CMV infection n = 57 [a]	Total n = 70
Genotype UL55			
1, n (%)	6 (46)	26 (46)	32 (46)
2, n (%)	2 (15)	12 (21)	14 (20)
3, n (%)	3 (23)	11 (19)	14 (20)
4, n (%)	2 (15)	3 (5)	5 (7)
5, n (%)	0 (0)	5 (9)	5 (7)
Genotype UL144			
1A, n (%)	4 (31)	10 (18)	14 (20)
1B, n (%)	1 (8)	2 (4)	3 (4)
1C, n (%)	0 (0)	1 (2)	1 (1)
2, n (%)	3 (23)	19 (33)	22 (31)
3, n (%)	5 (39)	25 (44)	30 (43)

a. In two postnatally infected infants only UL144 or UL55 could be genotyped

performed in 30 (52%) postnatally infected infants. All postnatally infected infants had a favourable outcome and normal hearing, determined between 16 months and eight years of life.

Genotype assignment

A total of 86 urine samples from 73 infants were included in the genotypic analysis. Genotyping was successful for both genes in 69 out of 73 (95%) infants, of whom 13 (19%) were congenitally infected and 56 (81%) postnatally infected. In addition, in two postnatally infected infants only UL144 or UL55 could be genotyped. In 13/71 (18%) infants, genotyping was performed on two sequential samples collected between one day and two months after the initial sample. In all cases genotype assignments were identical in both samples (data not shown).

The genotype distribution for UL55 and UL144 was similar for the congenitally and postnatally infected groups (Table 3). CMV UL55 genotype 1 and UL144 genotype 3 were the most prevalent genotypes. There were no differences in UL55 and UL144 genotypes with respect to development of lenticulostriate vasculopathy in both groups (data not shown).

Twenty four infants included in this study belonged to non-identical twins. Two infants (one twin) were congenitally infected, 14 infants (seven twins) postnatally. The remaining eight included postnatally infected infants were part of eight twins, of whom only one infant acquired CMV, and the other did not. All CMV infected infants of the twins were counted as an individual. A matching genotype for UL55 and UL144 in both infants belonging to twins was observed in 6/8 and 7/8 twins, respectively. One pair of twins had different genotypes for both UL55 and UL144, and another had different genotypes for UL55 only. These results were confirmed upon complete repetition of the analysis. The genotype distribution did not change when twins were excluded from analysis (data not shown).

Viral load

CMV load was determined in urine samples of all infants. The median viral load was significantly higher in congenital infection (6.34 Log_{10} copies/ml [IQR 1.62]) compared to postnatal infection (5.24 Log_{10} copies/ml [IQR 1.43]), p=0.002.

There were no statistical differences in viral load between UL144 genotypes, as shown in Figure 1. There was a trend towards an association between viral load and UL55 genotypes in both the congenitally infected group (p=0.052) and the postnatally infected group (p=0.057). UL55 genotype 1 was found in infants with lowest viral load. UL55 genotype 3 and 4 were identified in infants with congenital infection and the highest viral load.

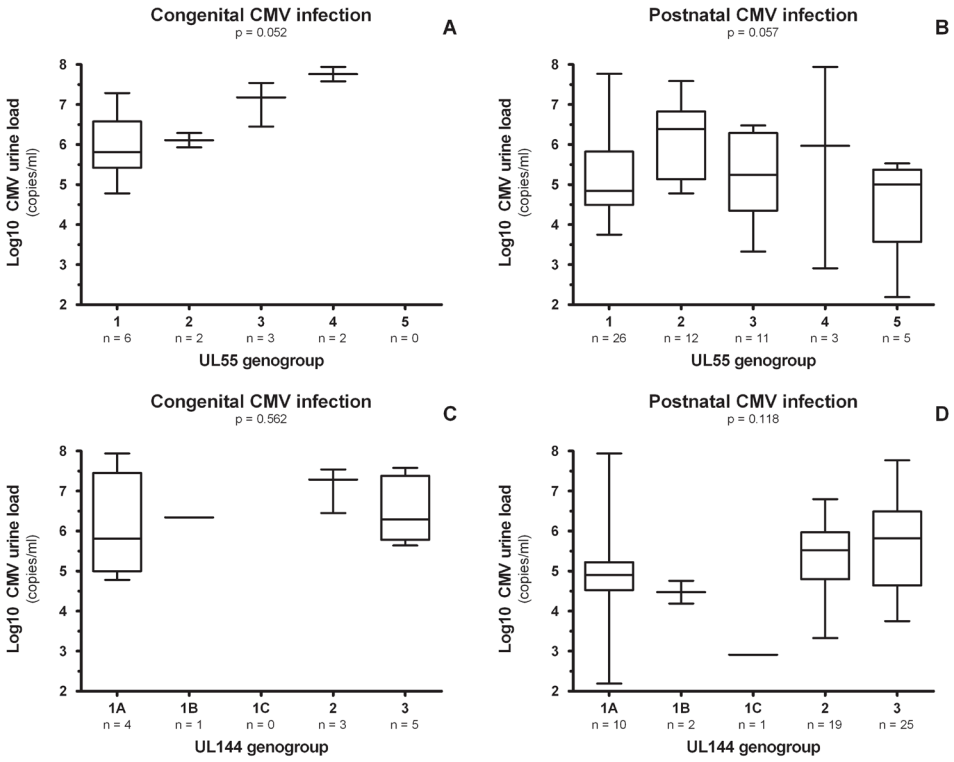

Figure 1. Log10 CMV urine load in congenitally and postnatally infected infants with respect to UL55 genotypes (A and B, respectively) and UL144 genotypes (C and D, respectively).

Bar in boxplot represents median viral load after log_{10} transformation. Upper and lower limit of boxplot represent 75th and 25th percentile, respectively. Whiskers represent full range.

Discussion

To our knowledge, this is the first study that assessed the prevalence of various genotypes in preterm infants with postnatal CMV infection, and examined the relationship between genotype and disease severity in both congenitally and postnatally infected infants. UL55 and UL144 genes have been demonstrated to be good candidates for genotyping, because of the significant genetic variation observed in these genes.[9,18]

The distribution of UL55 and UL144 genotypes among the infants with congenital infection in the present study is generally consistent with previously reported data.[9,11,21–23] UL144 genotype 3 is the predominant genotype (43% of all infants), but UL144 genotype 2 is much more represented in our population than in the cohort of Lurain et al.[9], 31.4% versus 9.0%, respectively. In accordance with other studies,[11,23–25] UL55 genotype 1 was the predominant genotype (46% of all infants).

Our study shows that the genotype distribution of CMV is similar whether they are transmitted vertically (intrauterine) or horizontally (most probably through breast milk) from mother to infant. Furthermore, we documented that the same CMV genotypes are able to cause severe CMV disease, sensorineural hearing loss and neurodevelopmental delay in congenitally infected infants and asymptomatic disease and normal outcome in postnatally infected infants. This may suggest that other factors, especially time of onset of the infection and hence, stage of brain maturation, are more important than CMV genotype with respect to development of severe disease.

According to recent studies,[26–28] the risk of development of sensorineural hearing loss or neurodevelopmental delay is increased significantly during early pregnancy compared to the third trimester of pregnancy. This may be related to the vulnerability of the developing brain during different stages of its maturation. Postnatally acquired CMV infection among preterm infants who are infected before they reach term-equivalent age may be comparable with third trimester congenital infection. Therefore, it is not surprising that the postnatally infected infants in our study and the three congenitally infected infants, in whom a third trimester infection was confirmed, had minor cerebral abnormalities and a favourable outcome, irrespective of CMV genotype.

UL55 genotype 3 was recently found to be associated with severe manifestations of CMV disease in congenitally infected infants.[11] Due to the small sample size in our congenitally infected group, we are not able to confirm these results. In contrast to a study by Yu et al.[21], none of the infants with UL55 genotype 1 and symptomatic CMV disease were diagnosed with CMV related liver disease. Several studies on congenital

CMV infection have suggested that blood and urine CMV loads may be important markers of CMV disease severity.[21,22,29–31] We have recently documented that infants with postnatal CMV infection have significantly lower urine CMV load than infants with congenital CMV infection.[32] In the current study, we have additionally shown that this difference consisted irrespective of genotype distribution. The previously suggested association between genotypes, high viral load and development of sequelae could not be confirmed in present study.[15,22]

This study included eight pair of twins, in which both twins had a CMV infection and eight infants belonging to a twin in which only one infant was infected. Genetic susceptibility for CMV and other viral infections in multiple pregnancies and twins has been documented previously.[33,34] It is of interest that one pair of twins had different UL55 and UL144 genotypes suggesting infection with different strains. The source of their infection could not be determined as maternal breast milk was not available. A contamination or mix up of urine samples was unlikely, while the complete analytical process was repeated and resulted in the same genotype assignments.

This study is limited by the small sample size of infants with congenital infection. Therefore, the relation between genotype, viral load, and severity of the disease in this group could not be analyzed statistically. Future studies in a larger population of infants infected within the same period of pregnancy (i.e. first or second trimester) should be designed to evaluate this association.

In conclusion, the CMV genotype distribution in severe congenital and asymptomatic postnatal CMV infection in preterm infants was similar, suggesting that other factors than viral genotype, especially stage of brain maturation are mainly responsible for the development of neonatal symptoms and adverse outcome. The role of viral load in relation to genotype and stage of brain development needs further study.

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6 Reduced occipital FA on cerebral diffusion tensor imaging in preterm infants with postnatally acquired CMV infection

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Abstract

Background

Detection of white matter (WM) abnormalities on MRI is important with regard to neurodevelopmental outcome in preterm infants. Long-term neurodevelopmental outcome of preterm infants with postnatal CMV infection has not been extensively studied.

Objectives

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

Methods

Preterm infants (<32 weeks gestational age - GA) who were admitted to our hospital between 2007 and 2010 and had cerebral DTI at term-equivalent age (TEA, 40 weeks GA), were included. CMV PCR in urine collected at TEA was performed to diagnose postnatal CMV infection. Congenital infection was excluded. Regions of interest were drawn in the frontal, parietal and occipital WM on which mean diffusivity (MD), 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 preterm infants and 61 non-infected 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 non-infected 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 between infected and non-infected infants.

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 non-infected controls.[4] However, a poorer cognitive and motor function in these infants compared to controls was documented at school age, although performance was within the normal range.[5]

It has been documented that 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 our best 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 magnetic resonance imaging (MRI) at term-equivalent age (TEA, 40 weeks postmenstrual age) correlate 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, and 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 all 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-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 posthaemorrhagic ventricular dilatation (PHVD) (n=6), intraventricular haemorrhage (IVH) grade 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 and clinical data were collected, as has been described previously.[2] In addition, the following cerebral ultrasonography (cUS) data were collected: IVH, periventricular echogenicity (PVE), lenticulostriate vasculopathy and germinolytic cysts at TEA. 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 one 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

The infants were sedated with 50-60 mg/kg oral chloral hydrate before the MRI examination. Throughout the examination a neonatologist was present, and heart rate, transcutaneous oxygen saturation and respiratory rate were monitored. Hearing protection was provided (Natus Minimuffs; Natus Medical Inc., San Carlos, USA). MR imaging 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 images and axial or coronal 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 x 1.44 x 2.0 mm; duration 4.32 mins), 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 >10 years 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 one (normal), two (mild abnormality) or three (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 DTIs 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

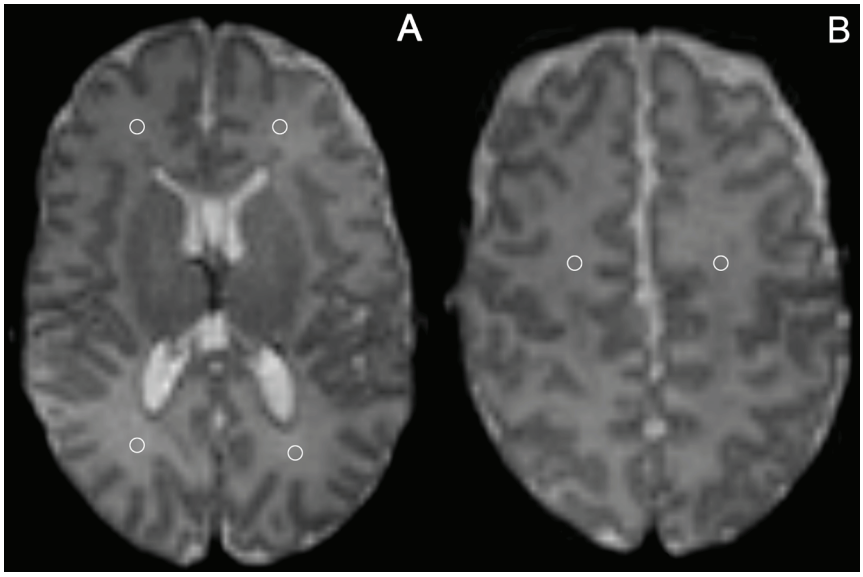

Figure 1. Example of drawn frontal and occipital ROIs (A) and parietal ROIs (B).
Circles represent ROIs of 15-18 voxels.

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 center 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. In our experience, 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 chi-square test. After analysis of data normality using Kolmogorov-Smirnov test, continuous variables were analyzed using non-parametric Mann-Whitney U test. In demographic and clinical characteristics, a 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 (axial, radial, and mean diffusivity, and FA in three regions) and therefore, $p < 0.004$ was considered statistically significant in axial, radial, and mean diffusivity, and FA.

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 one week after birth. In two infants (10%), a highly sensitive CMV

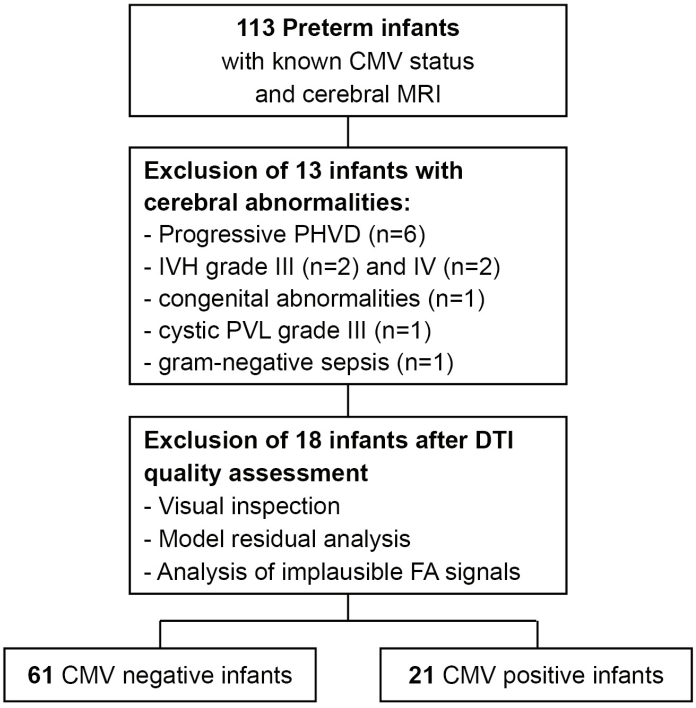

Figure 2. Flowchart.

CMV – cytomegalovirus; DTI – diffusion tensor imaging; IVH – intraventricular haemorrhage; MRI – magnetic resonance imaging; PHVD – posthaemorrhagic ventricular dilatation; PVL – periventricular leukomalacia

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 non-infected infants are shown in Table 1. Non-Dutch maternal ethnicity was significantly more frequent in postnatally infected infants compared to non-infected infants (9/21 [43%] and 8/61 [13%], p=0.004). All infants with a postnatally acquired CMV infection received breast milk compared to 71% of non-infected infants (p=0.005). Lenticulostriate vasculopathy was significantly more often present at TEA in postnatally infected infants compared to non-infected infants (8/21 [38%] and 7/21 [12%], p=0.007).

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

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

cPVL – cystic periventricular leukomalacia; IRDS – infant respiratory distress syndrome; LSV – lenticulostriate vasculopathy; PDA – persistent ductus arteriosus; TEA – term-equivalent age.

* Histopathologic examination of placenta was available in 17/21 (81%) CMV positive infants and 47/61 (77%) CMV negative infants.

White matter abnormalities

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

Table 2. 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
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

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 infants 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).

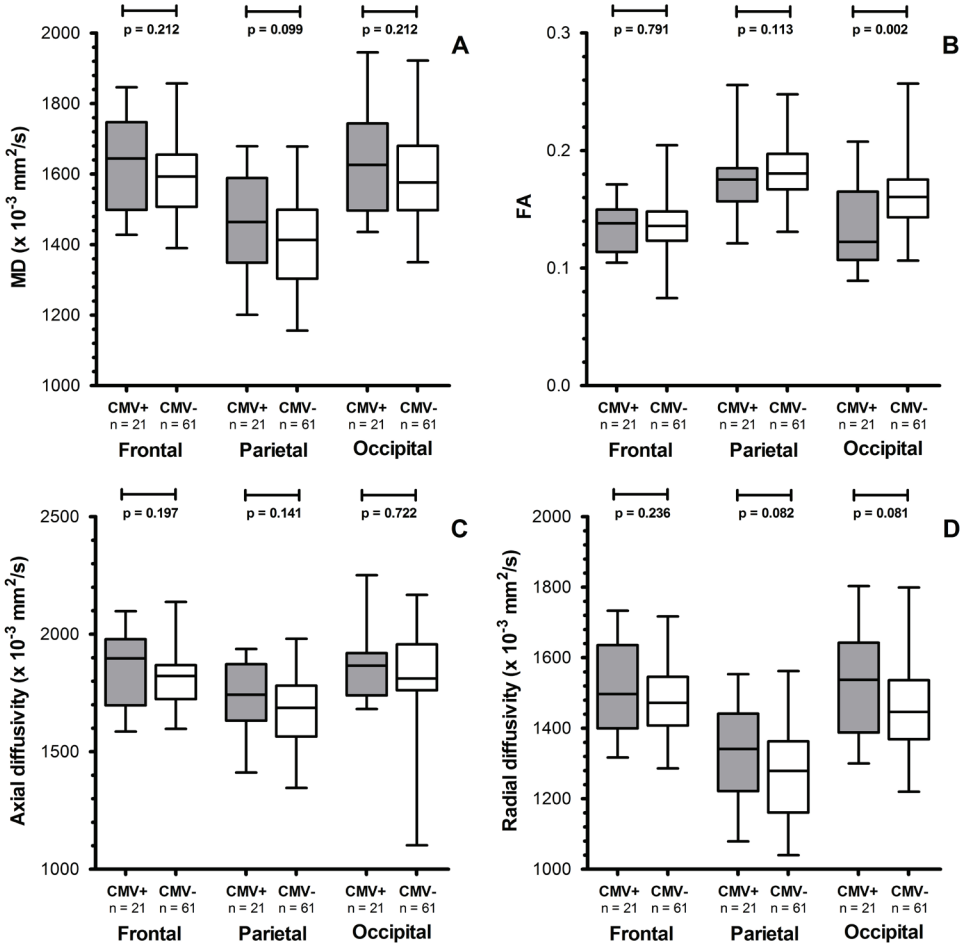

Figure 3. MD (A), FA (B), axial (C) and radial diffusivity (D) in frontal, parietal and occipital WM of infants with (CMV+) and without (CMV-) postnatal CMV infection.

Bars in boxplot represent median. Upper and lower limit of boxplot represent 25th and 75th percentile and whiskers represent full range.

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 postnatally infected. There were no significant differences in global developmental quotient between infected (median 99 [IQR 96-105]) and non-infected 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 non-infected 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, pre-myelinating 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 non-infected 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 previously documented 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 four infants with

periventricular leukomalacia suggesting loss of oligodendrocytes within the developing WM. However, neuro-imaging was performed at a mean age of three 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 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 non-infected infants was within the normal range.[5] Unfortunately, neuro-imaging was not performed in this study.

In present study we show that the neurodevelopmental outcome did not differ between infected and non-infected 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.

Breast milk is a known risk factor of postnatal CMV infection[1,2] and in our study, CMV infected infants were significantly more often fed with breast milk compared to non-infected 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 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 preterm infants with postnatal CMV infection.

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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7 Hearing in preterm infants with postnatally acquired cytomegalovirus infection

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Abstract

Cytomegalovirus (CMV) is an important cause of sensorineural hearing loss (SNHL) in children. In contrast to congenitally infected infants little is known about hearing in preterm infants with postnatal CMV infection. We studied the hearing in 64 preterm infants during the first year of life and in 18 during the second year of life. None of the infants developed SNHL.

Introduction

Preterm infants fed with breast milk of cytomegalovirus (CMV) seropositive mothers are at high risk of postnatally acquired CMV infection.[1] The majority of these infants do not develop any clinical signs and symptoms of CMV infection and therefore they can be only diagnosed with this infection by screening.[2] Since it is known that CMV is an important cause of sensorineural hearing loss (SNHL) in infants with congenital infection, questions rise regarding hearing of preterm infants who are infected after birth but before term age.[3] Data on hearing of preterm infants with postnatally acquired CMV infection are scarce and limited to small numbers of patients.[4–6]

We studied hearing in a large group of preterm infants with postnatally acquired CMV infection during the first and second year of life.

Materials and methods

Preterm infants (GA <34 weeks) admitted to the level 3 neonatal intensive care unit (NICU) of University Medical Center Utrecht, the Netherlands between April 2003 and December 2010 and diagnosed with postnatal CMV infection were included. The diagnosis of postnatal CMV infection was made at the time of CMV infection in symptomatic infants or at term-equivalent age by screening. The epidemiologic data from infants born after April 2007 were partly described previously.[2] The Internal Review Board of our hospital approved this study.

Postnatal CMV infection was determined by CMV PCR of urine collected at term-equivalent age during a visit to the follow-up clinic of asymptomatic infants. Symptomatic postnatal CMV infection was considered when the preterm infant presented with sepsis-like illness, thrombocytopenia and/or respiratory disease weeks after birth, had a negative bacterial blood culture, and positive CMV PCR of urine and plasma. Congenital CMV infection was excluded in all infants by negative CMV PCR of urine collected shortly after birth, or negative CMV PCR of dried blood spot filter cards in combination with anti-CMV IgM analysis, when early post partum urine was not available.[7] All infants were fed with fresh breast milk and leukocyte-depleted blood products were used for their treatment.

The following data were collected: gestational age, birth weight, gender, cerebral abnormalities documented by neuro-imaging, mechanical ventilation for more than 7 days, use of high frequency oscillation ventilation, persistent ductus arteriosus treated

with indometacin or surgery, use of loop diuretics or aminoglycosides and CMV urine load at term-equivalent age. Infants with congenital or chromosomal abnormalities were excluded.

Cranial ultrasonography (cUS) was performed in all infants on admission to the NICU and was repeated on a weekly basis until discharge. The presence of intraventricular haemorrhage, development of calcifications in basal ganglia such as lenticulostriate vasculopathy (LSV) or germinolytic cysts was evaluated as described previously.[2] Cerebral magnetic resonance imaging (MRI) was performed at term-equivalent age. Opthalmologic examination was performed in all infants.

In all studied infants hearing was tested in the neonatal period, before discharge from the NICU, using the automated auditory brainstem response (AABR) test (ALGO® 3i, Natus Medical Incorporated, San Carlos USA). To determine SNHL, auditory brainstem response audiometry (ABR) was performed during the first year of life and repeated during the second year of life. When ABR-testing could not be performed, for example when the infant was not silent enough, sound field behavioral-observation audiometry and tympanometry were performed to determine degree and type of hearing loss. SNHL was defined as a threshold elevation of >20dB without any indication of a conductive component of hearing loss.[8,9]

The neurodevelopmental outcome of included infants was estimated using the Griffiths mental developmental scale (GMDS), including the language sub-scale (C) at age of 18 months.

Results

During the study period 88 preterm infants were diagnosed with postnatally acquired CMV infection. One infant died before 6 months of age and three were lost to follow-up. Congenital infection was excluded in all infants. Characteristics of 84 studied infants of whom six developed symptoms of postnatal CMV infection are described in Table 1 and Table 2, respectively. In all infants chorioretinitis was excluded by opthalmologic examination. None of the 84 infants with postnatal CMV infection had hearing loss determined by AABR in the neonatal period. ABR-testing was performed in 64/84 (76%) infants during the first year of life (median corrected age of 7 months, range 2–11), of whom none developed SNHL. In 10/64 (16%) infants conductive hearing loss ≤30dB and in 3/64 (5%) >30dB was present due to middle ear disease.

Table 1. Characteristics of 84 infants with postnatal CMV infection of whom 64 infants had a hearing test during the first year of life and 18 infants had a hearing test during the second year of life.

Characteristics	Postnatal CMV infection n = 84	Hearing test in first year n = 64	Hearing test in second year n = 18
Gestational age, median, wk (range)	28.7 (24.3 – 33.9)	28.6 (25.0 – 33.9)	27.9 (25.0 – 33.9)
Gestational age ≤ 27 weeks, n (%)	26 (31)	21 (33)	8 (44)
Birth weight, median, g (range)	1055 (600 – 1950)	1043 (600 – 1950)	1038 (700 – 1950)
Male gender, n (%)	42 (50)	32 (50)	9 (50)
Symptomatic CMV infection, n (%)	6 (7)	5 (8)	2 (11)
LSV on cUS at TEA, n (%)	30 (35)	25 (39)	8 (44)
Germinolytic cysts on cUS at TEA, n (%)	12 (14)	10 (16)	1 (6)
IVH on cUS, n (%)	22 (26)	17 (26)	6 (33)
grade I, n (%)	5 (6)	4 (6)	1 (6)
grade II, n (%)	10 (12)	6 (9)	4 (22)
grade III, n (%)	4 (5)	4 (6)	0 (0)
grade IV, n (%)	3 (4)	3 (5)	1 (6)
Treated PHVD, n (%)	5 (6)	4 (6)	1 (6)
CMV load, median, copies/ml, (range)	3.3 x 10^5 (2.5 x 10^2 – 8.7 x 10^7)	3.3 x 10^5 (4.6 x 10^2 – 8.7 x 10^7)	3.3 x 10^5 (2.1 x 10^3 – 9.8 x 10^6)
>7 days of mechanical ventilation, n (%)	16 (19)	12 (19)	2 (11)
HFO ventilation, n (%)	10 (12)	7 (11)	3 (17)
PDA treated with Indometacin, n (%)	21 (25)	15 (23)	5 (28)
Hypotension treated with inotropes, n (%)	22 (26)	17 (27)	4 (22)
Use of loop diuretics, n (%)	3 (4)	3 (5)	1 (6)
Use of aminoglycosides, n (%)	80 (95)	61 (95)	17 (94)

CMV – cytomegalovirus; cUS – cranial ultrasonography; HFO – high frequency oscillation; IVH – intraventricular haemorrhage; LSV – lenticulostriate vasculopathy; PDA – persistent ductus arteriosus; PHVD – post-haemorrhagic ventricular dilation; TEA – term-equivalent age

Table 2. Characteristics of six infants with symptomatic postnatal CMV infection.

Infant / sex	GA (wks)	BW (g)	Delivery	Time of onset of illness (d)	Clinical diagnosis and laboratory abnormalities	Blood CMV load at onset of illness (copies/ml)	Urine CMV load at onset of illness (copies/ml)	cUS	MRI at TEA	Hearing in 1st and 2nd year
1/F	26	800	Vaginal	55	Pneumonia	6.7 x 10^4	6.8 x 10^5	Bilateral LSV at 2 months of age	NA	Normal
2/M	29	975	CS	106	Pneumonia	1.8 x 10^3	4.4 x 10^4	IVH grade II at birth, right-sided LSV at 4 months of age	Small infarctions in thalami	Normal *
3/M	27	1060	Vaginal	65	Thrombocytopenia	NA	3.3 x 10^4	Bilateral LSV at TEA	Mild dilatation of lateral ventricles	Normal
4/F	28	930	CS	42	Sepsis-like illness, thrombocytopenia	4.6 x 10^4	4.5 x 10^8	Bilateral GLC at TEA	GLC bilaterally	Normal *
5/M	28	985	Vaginal	34	Thrombocytopenia	NA	1.9 x 10^6	Left-sided small GLC at 2 months of age	Normal	Normal *
6/F	26	950	Vaginal	41	Sepsis-like illness	1.8 x 10^4	1.2 x 10^3	Bilateral GLC at TEA	Normal	Normal *

BW – birth weight; CMV – cytomegalovirus; CS – caesarean section; cUS – cranial ultrasonography; F – female; GA – gestational age; GLC – germinolytic cyst; LSV – lenticulostriate vasculopathy; M – male; MRI – magnetic resonance imaging; NA – not available; TEA – term-equivalent age

* 2nd year ABR not available, GMDS assessment performed

Subsequently, a hearing test was repeated in 18/84 (21%) infants during the second year of life (median corrected age of 33 months, range 12-50), of whom eight (44%) infants had ABR-testing and ten (56%) infants were tested using otoacoustic emission (n=4), sound field audiometry (n=3), play audiometry (n=3) and all combined with tympanometry. None of these infants had SNHL. Seven (39%) infants had conductive hearing loss due to middle ear disease.

In 58/84 (69%) infants the neurodevelopmental outcome was estimated using the GMDS including the language sub-scale at 18 months of uncorrected age (median 18.5 months, range 16.3-23.7). Median age corrected for prematurity was 15.8 months (range 13.0-21.0). The mean developmental quotient score estimated by GMDS corrected for prematurity was 104.4 (SD 9.9) and the mean score of the language subscale was 16.7 months (SD 2.1). The mean corrected developmental quotient score in 14/18 (78%) infants in whom SNHL was excluded during the second year of life was 102.9 (SD 7.9) and the mean score of the language subscale was 15.7 months (SD 1.5). Both scores were not different compared with scores of infants in whom hearing tests were not performed. Viral load in 17/18 (94%) infants of whom hearing was tested during the second year of life did not differ from viral load in 64/66 (97%) infants who were not tested at that time (median 3.3x10^5 copies/ml versus 3.1x10^5 copies/ml, p=0.8).

Discussion

This study shows that preterm infants with postnatally acquired CMV infection do not develop SNHL during the first two years of life.

CMV is an important cause of SNHL in children. In contrast to congenitally infected infants little is known about the hearing in preterm infants with postnatal CMV infection. Audiologic follow-up of infants with postnatal CMV infection has so far only been studied twice and was limited to small numbers of patients.[4–6] In the first study 30 postnatally infected preterm infants with gestational age between 35-37 weeks were tested at 3 years of age and all had normal hearing.[4] In the second study 22 infected preterm infants born before 32 weeks of gestation were evaluated at 2 and 4.5 years of age, respectively.[5] None of the infected infants developed SNHL. Furthermore, in 20 of these previously studied infants the hearing was tested again at school age and none of them developed SNHL.[6] This is in line with our study where we found no SNHL in 64 infected preterm infants during the first year of life and in 18 during the second year of life regardless of the presence of cerebral abnormalities, clinical symptoms of CMV

disease or urine CMV load. Moreover, the neurodevelopmental scores using the GMDS including language sub-scale were normal in all infants with and without hearing test at 18 months of corrected age. Since the language sub-scale of the GMDS score may not exclude mild sensorineural hearing deficit (such as auditory neuropathy), the hearing of infected infants will be assessed again at school age.

Recently, the relationship between postnatal CMV infection and development of SNHL (including auditory neuropathy) in a preterm infant was suggested.[10] This infant, born at a gestational age of 26 weeks developed CMV infection within 3 weeks of age and at term-equivalent age bilateral auditory neuropathy was diagnosed. At 4 years of age, her language perception was normal and her language expression was mildly delayed. The suggestion that extremely low birth weight infants who develop CMV infection within several weeks after birth are at high risk of developing auditory deficit cannot be confirmed in our study. None of 21 (25%) infants born ≤27 weeks of gestation and none of 5/6 symptomatic infants in whom hearing was tested (median GA 27.3 weeks, range 26.0-29.6) developed SNHL during the first year of life. In the second year of life, hearing of eight infants born ≤27 weeks of gestation and two symptomatic infants was tested, none of them had SNHL. It is of interest that the development of LSV which was documented in 35% of studied infants was not associated with hearing loss.

The exact mechanism of acquiring SNHL caused by CMV infection is still unknown; however it may be the result of persistent cochlear inflammation.[11] CMV has been recently isolated from the inner ear of a 15-months-old boy one month after a documented primary CMV infection.[12]

A limitation of our study is the limited number 18/84 (21%) of preterm infants with CMV infection that could be tested during the second year of life. All parents of non-tested infants were convinced that their children had no hearing deficits and therefore they refused a hearing test between 18 and 24 months of age. It is, however, likely that severe hearing impairment among infants who were not tested during the second year of life would have been recognized using GMDS and its language subscale. Furthermore, there were no differences in viral load and other hearing loss risk factors between tested and non-tested infants. Since progressive SNHL caused by CMV infection may sporadically develop in later life future evaluation of hearing in our population is still desirable. Neurodevelopmental outcome of infected and non-infected infants will be assessed in the near future.

In conclusion, postnatally acquired CMV infection among preterm infants is not related with SNHL during the first and second year of life.

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8 Neurodevelopmental outcome of preterm infants with postnatally acquired cytomegalovirus infection

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Abstract

Introduction

Long-term sequelae of postnatal CMV infection in preterm infants are insufficiently evaluated. The aim of this study was to assess whether postnatally acquired CMV infection in preterm infants affects their neurodevelopmental outcome.

Methods

Preterm infants (<32 weeks) treated in our NICU between January 2007 and December 2010 were included. Postnatal CMV infection was diagnosed at term-equivalent age, using CMV PCR in urine. Congenital CMV infection was excluded. Clinical, demographic and neuro-imaging data were collected. Neurodevelopmental outcome was assessed using Griffiths Mental Developmental Scales (GMDS) at 16 months and 24 months corrected age (CA), respectively, and Bayley Scales of Infant and Toddler Development-III (BSITD-III) at 24 months CA, as well as age of independent walking (AOIW). Differences in neurodevelopmental outcome between infected and non-infected infants were calculated.

Results

CMV status was determined in 449 infants of whom 390 (87%) and 326 (73%) were assessed at 16 months and 24 months CA, respectively. Sixteen percent of studied infants had a postnatal CMV infection. Infected infants had significantly lower gestational age, were more frequently born from non-native Dutch mothers and more often developed lenticulostriate vasculopathy compared to non-infected infants. At 16 months CA, infected infants performed better on the GMDS locomotor scale (p=0.049). They were also significantly younger able to walk unaided (p=0.026). Multivariable linear regression analysis showed that this difference was related to ethnicity. There were no differences between infected and non-infected infants at 24 months CA.

Conclusion

Postnatal CMV infection in preterm infants does not adversely affect neurodevelopmental outcome at two years CA.

Introduction

A postnatal cytomegalovirus (CMV) infection is common in very low birth weight (VLBW) infants with an estimated prevalence of 6 – 59%.[1,2] Breast milk from CMV seropositive mothers is the main source of postnatal CMV infection. Ninety-six percent of these mothers shed CMV in their breast milk after delivery due to local reactivation in the breast.[3] The great majority of postnatally infected infants does not present with symptoms and signs of CMV infection. Symptomatic disease such as sepsis-like illness with thrombocytopenia, pneumonia or hepatitis is very rare.[4] Risk of infection is associated with lower gestational age (GA).[4,5]

Data on long-term neurodevelopmental outcome of a postnatal CMV infection in VLBW infants are scarce and limited to small samples. In these studies, no neurodevelopmental sequelae of postnatal CMV infection were observed.[6–8] Recently, Bevot et al.[9] have shown once again that cognitive and motor function assessed at school age in 20 infants with postnatal CMV infection transmitted through breast milk was within normal range.However, in this case-control study the outcome of these infants was poorer than outcome of infants without CMV infection. Because of concerns regarding short- and long-term consequences of postnatal CMV infection and in the absence of conclusive studies on neurodevelopmental outcome of infected infants fresh breast milk is not always recommended for VLBW infants to prevent CMV acquisition.[10,11] Since breast milk is known to improve infant health outcome in both industrialized and developing countries,[12] it is important to study whether postnatally acquired CMV infection affects neurodevelopmental outcome in a large cohort of preterm infants.

In this cohort study we assessed the cognitive and motor development of preterm infants with postnatal CMV infection.

Methods

Study population

From April 2007 until December 2010, all preterm infants (<32 weeks GA) admitted to the Neonatal Intensive Care Unit (NICU) of the Wilhelmina's Children Hospital, Utrecht, The Netherlands were screened for presence of CMV in urine obtained at term-equivalent age (TEA, 40 weeks post-conceptional age) using CMV PCR, as described previously.[5] Congenital CMV infection was excluded using CMV PCR in urine obtained shortly after birth or through CMV PCR and anti-CMV IgM analysis of dried blood spot cards. Exclusion criteria were: absence of urine at TEA, severe intracranial lesions (i.e. porencephalic cyst at birth, cystic periventricular leukomalacia, post-haemorrhagic ventricular dilatation requiring insertion of a ventricular reservoir or ventriculo-peritoneal shunt), chromosomal anomalies, death before 16 months corrected age (CA), and no parental consent. The Internal Review Committee of our hospital approved this study.

The following clinical and demographic characteristics were collected: GA, birth weight, small for gestational age (SGA), gender, non-native Dutch maternal origin, Apgar scores at 1 and 5 minutes, acquisition of breast milk, respiratory distress syndrome, mechanical ventilation >7 days, chronic lung disease, persistent ductus arteriosus requiring treatment, use of inotropics, number of transfusions, sepsis, necrotizing enterocolitis, NICU admission days, and socio-economic status.

SGA was defined as birth weight by gestational age below the 10th percentile. Percentiles for our population were obtained from the Dutch perinatal registry.[13] Only fresh breast milk of their own mothers was used to feed the infants. The diagnostic criteria of respiratory distress syndrome, chronic lung disease, sepsis and necrotizing enterocolitis have been described previously.[5] Socioeconomic status was determined indirectly using socioeconomic status of the parent's neighborhood, and was provided by The Netherlands Institute for Social Research.[15] A score below -1 was considered low, between -1 and 1 average, and above 1 high socioeconomic status.

During admission to the NICU and at TEA, cranial ultrasonography was performed as described previously[5] and the following data were collected: intraventricular haemorrhage (IVH) graded according to Papile et al.,[14] lenticulostriate vasculopathy (LSV) and germinolytic cysts at TEA.

Symptoms of CMV disease included sepsis-like illness, pneumonia, cholestasis and/

or thrombocytopenia. The diagnosis of symptomatic CMV disease was made if these symptoms occurred weeks after birth in infants in whom bacterial sepsis was excluded by negative blood culture and the presence of CMV DNA was confirmed in urine and plasma at the time of symptoms.

Neurodevelopmental outcome

Neurodevelopmental outcome of the infants was assessed routinely at the outpatient clinic using the Griffiths Mental Developmental Scales (GMDS)[16] and Bayley Scales of Infant and Toddler Development-III (BSITD-III)[17] at 16 and 24 months CA, respectively. When BSITD-III could not be performed at 24 months, GMDS was used instead.

GMDS was assessed by neonatologists with >15 years experience in GMDS testing and consisted of five subscales: locomotor, personal and social, hearing and speech, eye and hand coordination, and performance. The outcome is expressed as a developmental quotient (DQ) of the subscales individually and a global DQ of the summed subscales. A global DQ of 100 is the mean of the general population with an SD of 12.

BSITD-III consists of five subtests which may be assessed independently. Because of time constraints only the cognitive and motor subtests were assessed by a special educator / pediatric physical therapist with >20 years experience in testing. The motor subtest includes scaled scores of fine motor and gross motor outcome. The cognitive subtest includes one cognitive scaled score. The composite scores of both cognitive and motor subtests were determined. A composite score of 100 is the mean of the general population with an SD of 15. Z-scores of both tests were calculated to compare outcome at two years of age. Both GMDS and BSITD-III scores were corrected for degree of prematurity.

When BSITD-III was assessed at 24 months CA parents were asked to provide the age of onset of independent walking (AOIW) defined as independently walking at least 5 steps,[18] expressed in months and weeks of age and corrected for prematurity. Furthermore, height, height-to-weight and head circumference were determined and converted in standard deviations according to the 1997 Dutch growth curves of The Netherlands Organisation for Applied Scientific Research TNO / Leiden University Medical Center.[19] An SD of ≤2 was considered severely delayed or growth retarded.

Statistical analysis

Statistical analysis was performed in PASW statistics® (version 18.0, SPSS Inc., 2009). 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 < 0.05 was considered statistically significant.

After analysis of neurodevelopmental outcome at 24 months CA with postnatal CMV infection (yes/no) as dependent variable, demographic and clinical parameters with a p-value <0.05 and severe IVH (grade IV) were analyzed to identify confounders. Multivariable linear regression analysis was performed with outcome results as dependent variable and postnatal CMV infection, significant parameters and IVH grade IV as independent variables.

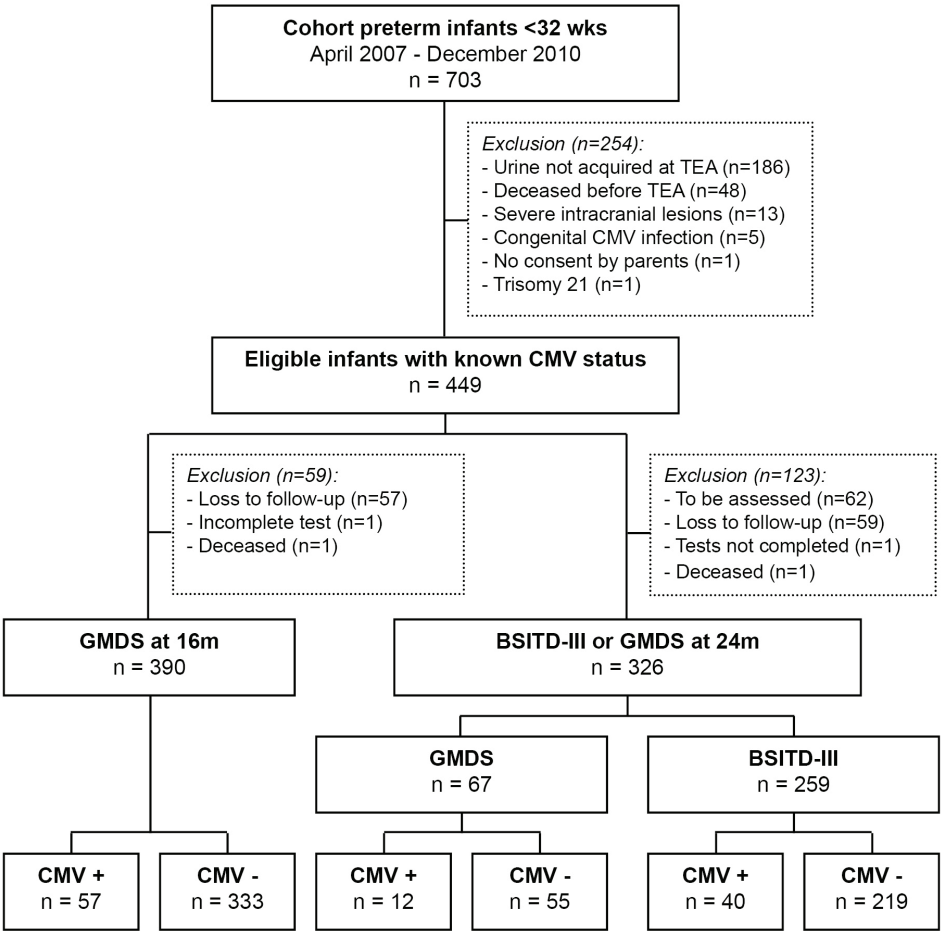

Figure 1. Inclusion of study population

BSITD-III – Bayley scales of infant and toddler development-III; GMDS - Griffiths mental developmental scales; TEA – term-equivalent age.

Results

Study population

Between April 2007 and December 2010, 449/703 (64%) preterm infants were eligible in whom urine was collected at TEA to perform CMV PCR (Figure 1). Postnatal CMV infection was diagnosed in 74 (16%) infants. Congenital infection was excluded in 63 (85%) infants using CMV PCR in urine acquired within three days after birth. In 11 (15%) infants, urine was not available and therefore, CMV PCR combined with anti-CMV IgM analysis of dried blood spots cards was performed to exclude congenital infection.

Four out of 74 (5%) infants had clinical and/or laboratory symptoms of CMV disease, including pneumonia (n=1), sepsis-like illness (n=2) and thrombocytopenia (n=1), and 70/74(95%) were diagnosed by screening of CMV DNA in urine at TEA. One infant with symptomatic CMV infection died at 6 months of age due to respiratory problems not associated with CMV and two symptomatic infants were lost to follow-up. None of the infants were treated with antiviral drugs.

Neurodevelopmental outcome was assessed in 390/449 (87%) infants at 16 months CA and 326/449 (73%) infants at 24 months CA. Demographic and clinical characteristics with respect to CMV infection of the infants assessed at 24 months CA are described in Table 1.

There were no significant differences in baseline characteristics between eligible and non-eligible infants (data not shown).

Neurodevelopmental outcome

GMDS at 16 months corrected age

Fifty-seven (15%) infants with postnatal CMV infection and 333 (85%) non-infected infants were tested at a mean of 16.3 months CA (SD 1.7) and 16.1 months CA (SD 1.4), respectively (Figure 1). The clinical characteristics of infants assessed at 16 months were comparable with characteristics of infants assessed at 24 months. Infected infants had significantly lower gestational age (p=0.007), were more frequently born from non-native Dutch mothers (p<0.001), and more often developed LSV at TEA (p<0.001) compared to non-infected infants. All CMV positive infants were fed with breast milk compared to 78% of the non-infected infants (p<0.001).

Table 1. Clinical and demographic characteristics of preterm infants assessed with BSITD-III or GMDS at 24 months CA with respect to postnatal CMV infection

	CMV positive infants n = 52	CMV negative infants n = 274	P
Clinical and demographic characteristics			
Gestational age, mean, wk (SD)	28.4 (1.8)	28.9 (1.6)	0.027
Birth weight, mean, g (SD)	1142 (301)	1205 (318)	0.189
SGA, n (%)	5 (10)	37 (14)	0.443
Male gender, n (%)	30 (58)	153 (56)	0.805
Non-native Dutch maternal origin, n (%)	29 (56)	36 (13)	< 0.001
Apgar at 1 min, mean (SD)	6 (3)	6 (2)	0.750
Apgar at 5 min, mean (SD)	8 (1)	8 (1)	0.536
Breast milk, n (%)	52 (100)	219 (80)	< 0.001
RDS, n (%)	19 (37)	135 (49)	0.092
Mechanical ventilation > 7 days, n (%)	6 (12)	50 (18)	0.240
Chronic lung disease, n (%)	0 (0)	11 (4)	0.142
PDA, n (%)	12 (23)	69 (25)	0.747
Use of inotropics, n (%)	13 (25)	105 (38)	0.067
Number of transfusion, mean (SD)	2 (3)	2 (3)	0.749
Sepsis, n (%)	13 (25)	92 (34)	0.225
Necrotizing enterocolitis, n (%)	0 (0)	8 (3)	0.212
NICU admission days, mean (SD)	32 (19)	34 (25)	0.586
Socio-economic status			
Low, n (%)	12 (23)	36 (13)	0.064
Average, n (%)	32 (62)	188 (69)	0.318
High, n (%)	8 (15)	50 (18)	0.621
Cranial ultrasonography findings			
IVH			
grade I, n (%)	6 (12)	34 (12)	0.861
grade II, n (%)	7 (14)	32 (12)	0.717
grade III, n (%)	3 (6)	9 (3)	0.383
grade IV, n (%)	3 (6)	11 (4)	0.567
LSV at TEA, n (%)	17 (33)	35 (13)	< 0.001
Germinolytic cysts at TEA, n (%)	7 (13)	34 (12)	0.834

IVH – intraventricular haemorrhage; LSV – lenticulostriate vasculopathy; PDA – persistent ductus arteriosus; RDS – infant respiratory distress syndrome; SGA – small for gestational age; TEA – term-equivalent age.

The mean locomotor quotient was significantly higher in infants with postnatal CMV infection than non-infected infants (Z-score 0.24 [SD 0.89] versus -0.04 [SD 1.01] which corresponds to a quotient of 100 [SD 13] versus 96 [SD 14], p=0.049) (Figure 2). Other subscales did not differ significantly. The mean global DQ was comparable in both infected and non-infected infants (101 [SD 9] versus 101 [SD 9], p=0.513, respectively) and was within the normal range.

When infants with IVH grade IV were excluded from the analysis a significant difference in locomotor quotient between infected and non-infected infants (mean 101 [SD 13] versus 97 [SD 14], p=0.045, respectively) was still present. No differences were found in other scales or global DQ.

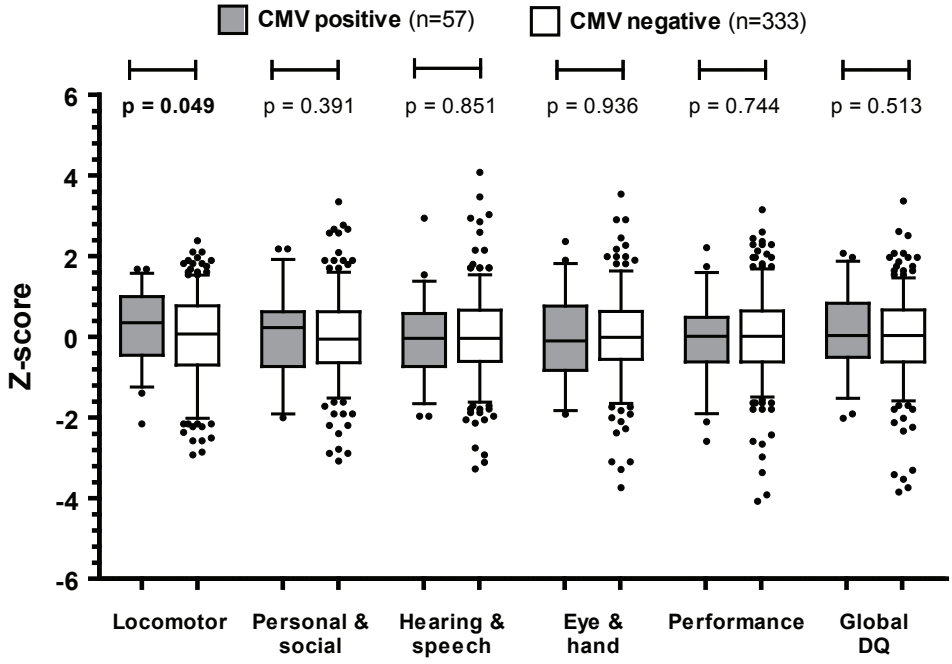

Figure 2. Z-scores of GMDS at 16 months corrected age in 57 postnatally infected infants and 333 non-infected infants.

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.

BSITD-III or GMDS at 24 months corrected age

Forty (15%) infants with postnatal CMV infection and 219 (85%) non-infected infants were tested using the BSITD-III at a mean of 24.9 months CA (SD 2.0) and 24.9 months CA (SD 2.2), p=0.892, respectively. In 12 CMV positive infants and 55 CMV negative infants the GMDS was used instead at a mean of 24.5 months CA (SD 1.4) and 25.3 CA (SD 1.7), p=0.147, respectively (Figure 1).

There were no significant differences in BSITD-III and GMDS Z-scores between postnatally infected and non-infected infants (Figure 3A and 3B, respectively). The mean corrected cognitive and total motor composite scores in infected and non-infected-infants (104 [SD 12] versus 104 [SD 12], p=0.992, and 110 [SD 9] versus 108 [SD 12], p=0.196, respectively) were within the normal range.

Mean AOIW was compared between 40 infants with postnatal CMV infection and 218 non-infected infants. Postnatally infected infants were significantly earlier able to walk compared to non-infected infants (14.7 months [SD 2.7] and 15.9 months [SD 3.1], p=0.026, respectively) (Table 2). Multivariable linear regression analysis including postnatal CMV infection, gestational age, non-native Dutch maternal ethnicity, LSV at TEA and IVH grade IV was performed to identify possible confounders (Table 3).

This analysis showed that significantly earlier onset of independent walking was related to non-native Dutch maternal ethnicity. Mean AOIW was 14.3 months [SD 2.9] in non-native infants compared to mean AOIW of 16.1 months [SD 2.9] in native infants (p<0.001). Infants with IVH grade IV had significantly later onset of independent walking compared to infants without IVH grade IV (mean 18.3 months [SD 2.5] versus 15.6 [SD 2.9], p=0.003, respectively). One CMV negative infant with IVH grade IV developed a hemiparesis.

There were no differences between postnatally infected infants and non-infected infants with respect to severely delayed growth, as shown in Table 2.

No differences in BSITD-III or GMDS results at 24 months CA were found when infants with IVH grade IV were excluded from the analysis. None of the Z-scores were significantly different between infected and non-infected infants. The mean corrected cognitive and total motor composite scores in infected and non-infected infants (106 [SD 10] versus 105 [SD 12], p=0.572, and 111 [SD 8] versus 108 [SD 12], p=0.073) were also within the normal range.

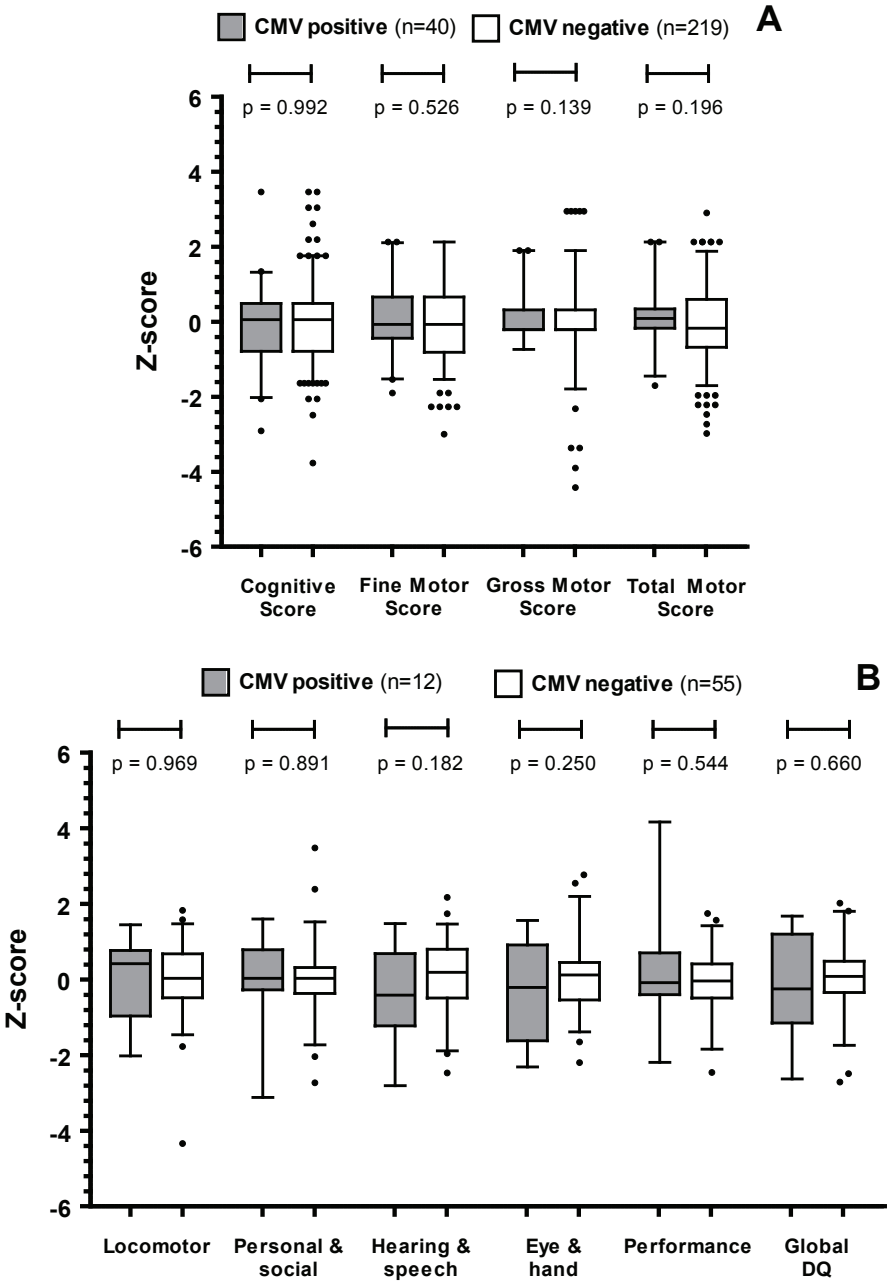

Figure 3. Z-scores of BSITD-III (A) at 24 months corrected age in 40 postnatally infected infants and 219 non-infected infants and GMDS (B) in 12 postnatally infected infants and 55 non-infected infants.

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. AOIW, ≤ 2 SD and ≥ 2 SD height, height-to-weight and head circumference at 24 months CA in postnatally infected and non-infected infants.

	CMV positive infants n = 40	CMV negative infants n = 218	P
AOIW, mean, months (SD)	14.7 (2.7)	15.9 (3.1)	0.026
Height ≤ 2SD, n (%)	5 (13)	20 (9)	0.513
Height-to-weight ≤ 2SD, n (%)	3 (8)	21 (10)	0.669
Head circumference ≤ 2SD, n (%)	1 (3)	6 (3)	0.928
Height ≥ 2SD, n (%)	0 (0)	2 (1)	0.543
Height-to-weight ≥ 2SD, n (%)	0 (0)	1 (1)	0.668
Head circumference ≥ 2SD, n (%)	0 (0)	3 (1)	0.456

AOIW – age of onset of independent walking; CA – corrected age.

Table 3. Multivariable logistic regression analysis of AOIW

Clinical and demographic parameters	Coefficient (ß)	SE	95% CI	P
Intercept	13.40	3.44		
Postnatal CMV infection	-0.33	0.58	-1.48 to 0.81	0.813
Gestational age	0.09	0.12	-0.15 to 0.33	0.326
Non-Dutch maternal origin	-1.56	0.53	-2.61 to -0.52	0.003
LSV at TEA	-0.07	0.51	-1.06 to 0.93	0.928
IVH grade 4	2.62	0.87	0.91 to 4.32	0.003

AOIW – age of onset of independent walking; CI – confidence interval; IVH – intraventricular haemorrhage; LSV lenticulostriate vasculopathy; SE – standard error; TEA – term-equivalent age

Discussion

This prospective study shows a favorable neurodevelopmental outcome in preterm infants with postnatal CMV infection. At 24 months CA, there were no significant differences in global neurodevelopmental outcome between infected and non-infected infants. Outcome data of preterm infants with a postnatal CMV infection are limited to small series and remained controversial.[20] In the first study, Paryani et al.[21] documented an increased incidence of severe neurologic sequelae (severe neuromuscular impairment, severe handicaps, developmental quotient <70) among 55 both term and preterm infants with postnatal CMV infection within the first two months of life. More recently Vollmer et al.[8] reported no differences in neurodevelopmental outcome of 22 infected infants and their non-infected controls at age 2.5 to 4 years. In the subsequent study by Bevot et al.[9] at a mean age of 8 years, neurodevelopmental outcome was again within the normal range. However, poorer motor and cognitive skills were reported in 20 postnatally infected infants compared to 21 non-infected infants. None of the reported studies used the GMDS or BSITD–III to assess neurodevelopmental outcome.

In the present study, the largest prospective cohort study to date, we did not show any adverse neurodevelopmental sequelae in 52 preterm infants with postnatal CMV infection at two years CA compared to 274 non-infected infants. It is of interest that CMV positive infants had a better gross motor performance at 16 months CA and had a significantly earlier onset of independent walking compared to non-infected infants. While the majority of CMV positive infants were non-native, mostly of Turkish and Moroccan ethnicity, we analyzed the association between postnatal CMV infection and AOIW for possible confounders and showed that non-native Dutch 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.(Nuysink, submitted)

The presence of IVH grade IV had a negative effect on onset of independent walking in the same multivariable regression analysis. While IVH grade IV may cause structural changes in cerebral tissue and therefore may affect neurodevelopmental outcome of preterm infants, we have analyzed the neurodevelopmental outcome at 16 and 24 months CA of infected and non-infected infants without this subgroup. Although the results of this analysis were comparable to the initial analysis including infants with IVH grade IV, both cognitive and motor scores were higher in both infected and non-infected infants after exclusion of infants with IVH grade IV.

Recently, Voss et al.[22] reported a significant association of lower maternal education,

grade III or IV IVH or PVL, and immigrant background of both parents with impaired long term composite intelligence quotient (10-13 years of age). No differences in early cognitive outcome between infected and non-infected infants were observed at both 16 and 24 months CA in our study. However, these differences may still manifest later in life. Therefore, neurodevelopmental outcome of the current cohort will be assessed again at five years of age.

Subtle cognitive and motor function impairment at 8 years, as observed by Bevot et al.[9], cannot be dismissed based upon our results, as the predictive value of BSITD-III on neurodevelopmental outcome at school age and later in life has not been definitely established yet. However, because of the study size, not all known risk factors of impaired neurodevelopmental outcome were addressed by Bevot et al.[9] and potential confounders like ethnicity may have biased their results. A future study with a sufficient sample size to identify possible confounders is needed to definitely establish neurodevelopmental outcome at school age.

Previously, we have shown that lenticulostriate vasculopathy is more common in preterm infants with postnatal CMV infection, a finding confirmed in the current study.[5] Moreover, we have documented the presence of microstructural changes in the occipital white matter of infected infants using diffusion tensor imaging.[(Nijman, submitted)] So far, both findings were not associated with impaired neurodevelopmental outcome at two years CA.

The results of the present study do not justify interventions like pasteurization, freezing or withholding breast milk to prevent CMV transmission in preterm infants. Positive effects of breast milk[23] are still likely to outweigh the possible adverse effects of a postnatal CMV infection.

The most important limitation of this study is the considerable number of infants that had to be excluded, mainly because urine could not be obtained at the visit to the outpatient clinic at TEA. However, demographic and clinical characteristics of tested and non-tested infants were not different at baseline, minimizing the risk of systemic bias.

In conclusion, postnatal CMV infection in preterm infants does not adversely affect neurodevelopmental outcome at two years corrected age. Since it is known that CMV disease may progress within several years after birth, a follow-up assessment at school age will be performed in the current study population.

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9

Summary and general discussion

Recommendations and future directions of research

Summary and general discussion

Early studies had indicated that CMV infection of preterm infants, often acquired after blood transfusion, could lead to severe disease and long-term sequelae.[1–3] This led to the recommendation for the use of screened or leucocyte-depleted blood to preterm infants.[4,5]

When Hamprecht et al.[6] reported that 96% of cytomegalovirus (CMV) seropositive mothers of preterm infants (<32 weeks of gestation) shed CMV in their breast milk and may infect their infants, several studies were carried out to investigate and reduce the risk of this breast-milk associated CMV infection in preterm infants. In many countries, preventive measures like freezing or pasteurization of breast milk were recommended for management of preterm infants admitted to the Neonatal Intensive Care Unit (NICU).[7,8] However, breast milk is essential to prevent prematurity-associated disease and promote growth, and should therefore be encouraged in preterm infants.[9] Furthermore, neurodevelopmental outcome and hearing of postnatally CMV infected infants has been studied insufficiently. Only one case-control study performed in 22 preterm infants with postnatal CMV infection was reported previously.[10] There are no data available on postnatally acquired CMV infection in the Netherlands.

In this thesis, we have described the epidemiology, risk factors, neuro-imaging, neurodevelopmental outcome and hearing in a large cohort of preterm infants with postnatal CMV infection.

Epidemiology and risk factors of a postnatal CMV infection

In **Chapter 2** we prospectively studied a cohort of preterm infants admitted to our NICU from April 2007 until June 2009. CMV PCR in urine collected at term-equivalent age (TEA) during a visit to the follow-up clinic, was used to diagnose postnatal CMV infection. Congenital infection was excluded in CMV positive infants by CMV PCR in urine, collected shortly after birth.

A relatively large number (112/507 [22%]) of infants was not eligible for inclusion in our study, because urine could not be collected at TEA. For those included, demographic and clinical data were collected and cranial ultrasonography (cUS) results, including germinolytic cysts and lenticulostriate vasculopathy (LSV) were evaluated.

Thirty-nine of 315 (12%) screened preterm infants acquired postnatal CMV infection, of whom the majority (33/39 [85%]) did not develop symptoms of CMV disease. Using multivariable logistic regression analysis, non-native Dutch maternal origin (OR 9.6

[95% CI 4.3-21.5]), gestational age (OR 0.7 [95% CI 0.5-0.9]) and breast milk (OR 13.2 [95% CI 1.7-104.5]) were determined to be independent risk factors of postnatal CMV infection. Furthermore, we documented that LSV at TEA was associated with postnatal CMV infection (OR 4.1 [95% CI 1.9-8.8]).

To date, this study describes the largest cohort of preterm infants screened for postnatal CMV infection worldwide. Our results showed that, in the Netherlands, postnatal CMV infection is common (12%) among preterm infants admitted to the NICU. Infants with low gestational age fed with fresh breast milk from non-native Dutch mothers are at greatest risk of postnatal CMV infection. In contrast to a study by Maschmann et al.[11] who reported that 16/33 (48%) infants with postnatal CMV infection were symptomatic (sepsis-like symptoms, liver involvement, myoclonias of arms and legs, neutropenia, and thrombocytopenia) the great majority (85%) of infants in our study were asymptomatic. This is in accordance with the results described in a recent systematic review by Kurath et al.[12], who reviewed 26 articles on postnatal CMV infection in preterm infants were reviewed, and the mean risk of symptomatic infection was estimated at 3.7% (range 0-34.5%).

As CMV is a neurotropic virus and may cause cerebral abnormalities, it was of interest to study neuro-imaging of infected preterm infants.[13] These infants are infected before they reach TEA and therefore, postnatal CMV infection may be comparable with third trimester congenital infection. In this chapter, we reported cUS results in all studied infants. The development of LSV occurred more often in infants with postnatal CMV infection compared to infants without infection. LSV has been associated previously with sensorineural hearing loss (SNHL) in congenitally infected infants.[14] However, in postnatally infected preterm infants, a relation of LSV with neurodevelopmental outcome was not established. Our findings called for further research with respect to cerebral MRI, hearing, and neurodevelopmental outcome in these infants, which is described in chapter 6, 7 and 8, respectively.

Mother to-infant CMV transmission

The risk of CMV transmission in preterm infants varied in different studies between 6-59%.[12] In Chapter 2, we have reported that 12% of all infants born <32 weeks of gestation were infected. We have also shown that 23/65 (35%) of infants born from non-native Dutch mothers, of whom the great majority is CMV seropositive, were infected with CMV.[15] The determinants associated with mother-to infant CMV transmission are still not sufficiently elucidated. It has been suggested that a high maternal anti-CMV

IgG level may be associated with an increased risk of CMV transmission in preterm infants.[16,17] To evaluate this association, we studied maternal anti-CMV IgG level of 79 seropositive mothers and their 94 infants (**Chapter 3**). Thirty-nine of 94 (41%) studied infants acquired a postnatal CMV infection. We did not find an association between absolute anti-CMV IgG levels in mothers and infants and risk of postnatal CMV infection. However, when we calculated the anti-CMV IgG infant-mother ratio (anti-CMV IgG level of the infant divided by the level of the mother) in all infants and analyzed the results with respect to CMV infection in 3 groups of infants (≤27 weeks of gestation, 28-29 weeks of gestation and ≥30 weeks of gestation), we found that a lower ratio increased the risk of postnatal CMV infection.

This suggests that mothers with high anti-CMV IgG levels, reflecting recent and/or frequent CMV reactivation or reinfection, may easily transmit CMV to their infants with low anti-CMV IgG levels. Infants with low gestational age and infants in whom transplacental transport of anti-CMV IgG is disturbed could therefore be at risk of postnatal CMV infection. Due to the small number of patients with symptomatic CMV infection we were not able to study the ratio in these subgroups.

Recently, studies on the use of anti-CMV hyperimmune globulin for prevention[18] or treatment[19] of postnatal CMV infection in preterm infants were published. Our results may support the use of anti-CMV hyperimmune globulins in selected cases.

Severity of CMV disease in relation to viral load and CMV genotype

As we have shown in Chapter 2, the great majority of postnatally infected preterm infants do not develop any symptoms of CMV infection and may be only diagnosed with this infection through screening. In contrast to these infants, the majority of congenitally infected infants admitted to the NICU, presented with severe clinical manifestations. These infants may subsequently suffer from SNHL, impaired neurodevelopmental outcome or even death. Several studies have shown that the presence of severe manifestation of CMV disease may be related to CMV load in blood or urine. Symptomatic infants had higher viral loads compared to asymptomatic infants.[20–22] Furthermore, the development of SNHL was also associated with higher viral loads.[23,24] In our study on viral load (**Chapter 4**), we compared the CMV load (CMV DNA copies per milliliter) in urine using CMV PCR in 17 infants with congenital infection and 45 infants with postnatal infection. This was the first study on viral load in preterm infants with postnatal CMV infection. CMV load in infants with congenital CMV infection was significantly higher compared to postnatally infected infants which may suggest that

a more extensive replication of the virus is responsible for the development of severe disease in congenitally infected infants.

A relationship between various CMV genotypes and manifestations of CMV disease or outcome have been reported in several studies on congenital CMV infection.[25–27] We were the first to determine the CMV UL55 and UL144 genotype distribution in 58 preterm infants with postnatal CMV infection (**Chapter 5**). In addition, we compared these results with the genotype distribution in 13 congenitally infected infants. After DNA extraction from urine of the infected infants, amplification and sequencing of CMV DNA was performed to determine the UL55 and UL144 genotype. Furthermore, clinical and neuro-imaging data was collected in all infants and correlated with CMV genotype. The results showed that the genotype distribution in congenital and postnatal infection was similar suggesting that other factors than virus genotype were responsible for disease severity. Similar to our study on viral load (Chapter 4) infants with congenital CMV infection had significantly higher CMV load compared to postnatally infected infants. Analysis of viral load in relation to genotype distribution for UL144 and UL55 did not show any differences in both groups.

As postnatal CMV infection in preterm infants born before 32 weeks of gestation, who acquire CMV before term-equivalent age, may be comparable to a third trimester congenital CMV infection and severely affected congenitally infected infants are mostly infected within the first or early second trimester, the timing of infection and stage of brain maturation may account for disease symptoms and adverse outcome. Due to the small sample size and selection bias of infants with congenital infection, the association between viral load, genotype and outcome of infants born within the first or second trimester could not be investigated.

It is of interest that one pair of twins had different UL55 and UL144 genotypes suggesting infection with different strains. The source of infection could not be determined, as breast milk was not available. Furthermore, eight infants belonging to a twin in which only one infant was infected, were included in this study. Differences in genetic susceptibility for CMV in a multiple pregnancy have been suggested previously.[28]

Neuro-imaging of preterm infants with postnatal CMV infection

Neuro-imaging of postnatally infected preterm infants has not been reported previously. Pre-myelinating oligodendrocytes are actively developing during the third trimester of pregnancy and may easily be adversely affected through infection, inflammation or hypoxia, resulting in impaired myelination and subsequent white matter (WM)

damage.[13] Using in vitro studies, it has been shown that CMV may infect most central nervous system cells, including oligodendrocytes.[29] We were the first to describe the development of LSV in postnatally CMV infected infants by cUS.[30] In Chapter 2, we reported that LSV at TEA was significantly more often present in infants with postnatal CMV infection compared to non-infected infants. We have also reported the development of germinolytic cysts in these infants without a history of subependymal haemorrhage in the early neonatal period. However, this finding was not significantly different between infected and non-infected infants.

In addition to cUS, magnetic resonance imaging (MRI) is often used to detect white matter (WM) injury, with cUS being useful in those with cystic WM injury and MRI in those with subtle WM lesions. The relationship between WM abnormalities detected at TEA by MRI and adverse neurodevelopmental outcome in preterm infants has been established previously.[31] In Chapter 4 and 5, we used MRI in postnatally infected preterm infants, and did not find gross WM abnormalities. Therefore, we used diffusion tensor imaging (DTI) in which the velocity and direction of water diffusion is used to determine microstructural WM changes (**Chapter 6**). Results are expressed in several parameters, including mean diffusivity (MD) and fractional anisotropy (FA). We have assessed these parameters in three cerebral regions of postnatally infected infants (n=21) and non-infected infants (n=61), with similar gestational age and birth weight. The FA in WM of infants with postnatal CMV infection was significantly lower in the occipital region, compared to the same region in non-infected infants. While MD in this region (and the other regions) did not differ, this may indicate microstructural WM changes in the occipital region of postnatally infected infants. To investigate whether these changes were significant with respect to neurodevelopmental outcome at 16 months corrected age, we used the Griffiths Mental Developmental Scales (GMDS) in the majority of infected (19/21 [90%]) and non-infected infants (60/61 [98%]). There were no differences in neurodevelopmental outcome. Therefore, microstructural WM changes in the occipital region of postnatally infected infants appear not to be related with impaired neurodevelopmental outcome at 16 months.

Neurodevelopmental outcome including visual performance should be assessed at a later age in these infants, to investigate the clinical significance of the reported microstructural WM changes.

Long-term outcome of postnatal CMV infection

Studies on the long-term outcome of preterm infants with postnatal CMV infection are still limited. Severely impaired neurodevelopmental outcome and SNHL has been reported in congenitally infected term infants.[23,32–35] Therefore, questions rise regarding the long-term outcome and hearing of postnatally infected preterm infants, who are infected after birth but before TEA. Reports on the hearing of postnatally infected preterm infants are scarce and limited to small numbers of patients.[10,36] In our study, auditory brainstem response (ABR) audiometry was used to examine hearing in 64 preterm infants with postnatal CMV infection in the first year of life and 18 infants in the second year of life (**Chapter 7**). In this large cohort of postnatally CMV infected infants, none of the infants developed SNHL. However, as most parents were convinced their infants did not have hearing loss, the loss of follow-up in the second year of life was high. Therefore, we used the language subscale of GMDS at 16 months corrected age to compare the hearing of 14 infants in which hearing tests were normal, with 44 infants who were not tested. There were no differences in global developmental quotient and language development, which was suggestive that there was no severe SNHL in the 44 infants who were not tested by the audiologist.

Data on the neurodevelopmental outcome of postnatally infected preterm infants is still very limited. Only two consecutive case-control studies by Vollmer et al.[10] and Bevot et al.[37] reported normal cognitive and motor outcome in 20 preterm infants with postnatal CMV infection. Following our previous studies, described in chapter 2, 6 and 7, we prospectively assessed neurodevelopmental outcome from 2009 onwards, using two neurodevelopmental outcome tests (**Chapter 8**). At 16 months corrected age GMDS was performed in 390 preterm infants, of whom 57 (15%) acquired postnatal CMV infection. Subsequently, at 24 months corrected age, the Bayley Scales of Infant and Toddler Development III (BSITD-III) test was performed in 328 preterm infants, of whom 53 (16%) had acquired postnatal CMV infection. In another 12 CMV positive infants and 55 CMV negative infants GMDS at 24 months corrected age was used instead. None of the infants with postnatal CMV infection were treated with antiviral medication or anti-CMV antibodies.

It is of interest that at 16 months corrected age, postnatally infected infants had better gross motor development compared to non-infected infants. There were no differences regarding general development. Using the more detailed BSITD-III, no differences were found in motor and cognitive development at 24 months corrected age. Moreover, postnatally infected infants showed a significantly earlier onset of independent

walking compared to non-infected infants. Subsequent multivariable regression analysis showed that ethnicity rather than postnatal CMV infection could explain the reported differences.

As in Chapter 2, an increased incidence of LSV was documented in postnatally infected infants. Development of LSV was not associated with impaired neurodevelopmental outcome.

While neurodevelopmental outcome was assessed in only one out of four symptomatic infants and was normal, it remains unclear whether symptomatic infants have a worse neurodevelopmental outcome compared to asymptomatic infants.

Prevention and treatment

In Chapter 2, 7 and 8 we have shown that postnatal CMV infection in preterm infants is usually asymptomatic and does not lead to hearing problems or neurodevelopmental delay at 2 years of age. Therefore, preventive interventions like freezing, pasteurization or withholding of breast milk do not seem to be necessary. The benefits of feeding preterm infants with breast milk likely outweigh the risk of symptomatic postnatal CMV infection or impaired neurodevelopmental outcome.[9,12,38] None of the infants with postnatal CMV infection in our study has been treated with antiviral medication or intravenous anti-CMV antibodies and the CMV disease was self-limiting in all symptomatic cases. The safety and efficacy of antiviral medication (Ganciclovir® and Valganciclovir®) has not been determined in preterm infants. Intravenous administration of anti-CMV antibodies may be helpful in symptomatic infants, but its efficacy has also not been studied sufficiently.[19] Treatment of postnatally infected preterm infants should only be considered in cases with life–threatening disease.

Conclusions

The following conclusions can be drawn from this thesis:

- The most important independent risk factors of postnatally acquired CMV infection among preterm infants are non-native Dutch maternal origin, breast milk and low gestational age (**Chapter 2**)
- The far majority (85%) of infected preterm infants does not develop any clinical symptoms of postnatal CMV infection, but one-third of infected infants developed lenticulostriate vasculopathy in the postnatal period (**Chapter 2**)
- An increased risk of postnatal mother-to-infant CMV transmission is also related to a low anti-CMV IgG infant-mother ratio (**Chapter 3**)
- CMV load in urine is significantly higher in infants with (symptomatic) congenital CMV infection compared to postnatally infected infants (**Chapter 4**)
- The UL55 and UL144 genotype distribution is similar in congenital and postnatal CMV infection, suggesting that not the viral genotype but the stage of brain maturation is responsible for development of severe disease (**Chapter 5**)
- Fractional anisotropy seen on DTI is significantly lower in the occipital region of postnatally infected preterm infants compared to non-infected infants at term-equivalent age, suggesting focal microstructural changes (**Chapter 6**)
- Postnatal CMV infection in preterm infants is not related with sensorineural hearing loss during the first and second year of life (**Chapter 7**)
- Postnatal CMV infection in preterm infants does not affect neurodevelopmental outcome at two years corrected age (**Chapter 8**)

Recommendations and future directions of research

1. Postnatal development of LSV (or germinolytic cysts) on cUS in preterm infants fed with fresh breast milk may suggest a postnatal CMV infection. A CMV PCR test in urine should be considered in these infants to confirm this infection. In preterm infants who develop sepsis-like illness and/or thrombocytopenia during the postnatal period and in whom bacterial blood culture remains negative, postnatal CMV infection should be considered.
2. The presence of low anti-CMV IgG infant-mother ratio is associated with an increased risk of postnatal CMV infection. Therefore, prophylactic administration of anti-CMV hyperimmune globulin in a selected population might eventually prevent postnatal CMV infection in most vulnerable preterm infants. Similarly, administration of anti-CMV antibodies in infants with symptomatic disease may have additional value in the treatment of postnatal CMV infection. The efficacy and safety of this preventive and therapeutic measure should be investigated in the future.
3. CMV genotype distribution in severe congenital and asymptomatic postnatal CMV infection in preterm infants is similar, suggesting that other factors than viral genotype, especially stage of brain maturation are primarily responsible for the development of severe disease. The role of viral load in relation to genotype and stage of brain development needs further study in a larger population of infants infected within the same trimester of pregnancy.
4. CMV transmission from mother to infant may differ between infants born after multiple pregnancy. While one infant acquires a postnatal CMV infection, the other infant may remain non-infected. Further research should investigate the role of genetic predisposition and susceptibility to CMV infection in twins.
5. The collection of urine in infants is difficult and time-consuming. The recent development of a CMV PCR on saliva provides a good alternative to urine, while saliva is easy to collect by a mouth swab. In congenitally infected infants, CMV PCR in dried saliva has shown to have high sensitivity and specificity for the diagnosis of congenital CMV infection. It would be interesting to study this method for diagnosis of postnatal CMV infection.
6. Congenital CMV infection may lead to sequelae even years after birth. However, there are very limited data on long-term outcome of preterm infants with postnatal CMV infection. Therefore, in the infants studied in this thesis, neurodevelopmental

outcome, including visual performance tests, and hearing need to be assessed at 5.5 years of age.

7. Congenital CMV infection in early pregnancy may lead to severe sequelae. As CMV vaccines are still not available, more attention should be paid worldwide to education and counselling on behaviour and hygienic measures in the home situation of pregnant women, to prevent CMV transmission especially within the two first trimesters of pregnancy.

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Nederlandse samenvatting

Cytomegalovirus

Het cytomegalovirus (CMV) is een van de grootste DNA-virussen en wordt zoals de meeste virussen in de Herpesvirus familie gekenmerkt door latentie: de mogelijkheid om na besmetting aanwezig te blijven in het lichaam. Deze latentie bestaat afwisselend uit een fase van chronische verspreiding en een stille fase, waarin het virus circulair als 'episoom' in de kern van een lichaamscel aanwezig is. Hierbij vindt geen uitwisseling plaats tussen DNA van het virus en DNA van de cel en er is ook geen sprake van verspreiding van het virus. Door nog onvolledig opgehelderde mechanismes wordt het virus met enige regelmaat geactiveerd en is er sprake van chronische verspreiding van het virus. De verspreiding van het virus kan plaats vinden via alle lichaamsvloeistoffen waaronder speeksel, bloed en urine. Pasgeborenen kunnen op meerdere manieren geïnfecteerd raken met CMV en daarvan nadelige gevolgen ondervinden.

Congenitale CMV infectie

Zwangere moeders die nog niet eerder met CMV in aanraking zijn geweest (seronegatieve vrouwen), kunnen geïnfecteerd raken tijdens de zwangerschap. Dit wordt een aangeboren of congenitale CMV infectie genoemd. Ook kinderen van moeders die al eerder een infectie hebben doorgemaakt, kunnen door reactivering van het virus of besmetting met een andere CMV-stam tijdens de zwangerschap een congenitale CMV infectie krijgen. Met name de pasgeborenen van moeders die voor het eerst in aanraking komen met CMV in het eerste trimester van de zwangerschap lopen een groot risico op negatieve gevolgen bij de geboorte (o.a. ernstige cerebrale afwijkingen, microcefalie en ernstig gehoorsverlies). Ook congenitaal geïnfecteerde kinderen zonder symptomen bij de geboorte, hebben later een verhoogd risico op ondermeer gehoorsverlies en slechte neurologische ontwikkeling. In Nederland is er bij ongeveer 5 op 1000 zwangerschappen sprake van een congenitale CMV infectie, waarbij 10% van de pasgeborenen symptomen heeft bij de geboorte. Van de 90% niet symptomatische kinderen heeft 10-15% kans op late symptomen van een congenitale CMV infectie zoals gehoorsverlies.

Er is nog geen definitieve behandeling voor een congenitale CMV infectie. Antivirale middelen worden gebruikt om bij symptomatische pasgeborenen het risico op gehoorsverlies te verkleinen. Bijwerkingen van deze middelen op latere leeftijd moeten nog worden onderzocht. Vaccinatie van seronegatieve vrouwen voor de zwangerschap,

zou een mogelijkheid zijn om een congenitale CMV infectie te voorkomen. Echter, omdat het eiwitkapsel van CMV continue verandert, is er nog geen effectief vaccin ontwikkeld. Met name preventieve hygiënische maatregelen tijdens de zwangerschap zijn belangrijk in het voorkomen van congenitale CMV infecties.

Postnataal verworven CMV infectie

Dit proefschrift richt zich voornamelijk op een tweede mogelijkheid waarop pasgeborenen een CMV infectie verwerven. Dit is een CMV infectie die na de geboorte plaats vindt, oftewel een postnatale CMV infectie. De eerste studies naar CMV infecties in prematuur geboren kinderen, die destijds meestal werden verworven via een bloedtransfusie, toonden aan dat CMV ernstige klinische gevolgen kan hebben op de korte en lange termijn. Sindsdien is het wereldwijd aanbevolen om bloed te gebruiken wat getest is op de afwezigheid van CMV of bloed zonder leukocyten (witte bloedcellen).

In 2001 werd aangetoond dat 96% van de moeders van prematuur geboren kinderen (minder dan 32 weken zwangerschapsduur) die eerder tijdens hun leven besmet waren met CMV (seropositief), het virus uitscheidt via de borstvoeding. Verder werd aangetoond dat de CMV positieve moeders via deze route hun kinderen met CMV kunnen infecteren. Nadat dit bekend was geworden, werden er verschillende studies uitgevoerd naar de risico's van een dergelijke infectie en op welke manier deze risico's beperkt konden worden. Sindsdien zijn er in veel landen preventieve maatregelen genomen om een CMV infectie via de borstvoeding bij de prematuur geboren kinderen op de Neonatale Intensive Care Unit (NICU) te voorkomen, zoals het invriezen of pasteuriseren van de borstvoeding. Zowel het invriezen als het pasteuriseren van de borstvoeding gaat echter ten koste van de kwaliteit. Met name in prematuur geboren kinderen is borstvoeding essentieel om ziekte die gerelateerd is aan prematuriteit te voorkomen en de groei te bevorderen. Daarom wordt borstvoeding bij deze kinderen door de WHO sterk aanbevolen. Het is de vraag of de nadelige effecten van het invriezen, pasteuriseren of zelfs onthouden van de borstvoeding opwegen tegen het voorkomen van een postnataal verworven CMV infectie.

Langetermijneffecten van een postnataal verworven CMV infectie zijn onvoldoende onderzocht. Er is slechts één case-control studie waarin de ontwikkeling van 22 CMV positieve en negatieve prematuur geboren kinderen prospectief met elkaar wordt vergeleken over een tijdsspanne van acht jaar. Hierin wordt een normale ontwikkeling gezien van zowel geïnfecteerde als niet geïnfecteerde kinderen. Er zijn geen

Nederlandse studies gepubliceerd over de effecten van een postnataal verworven CMV infectie in prematuur geboren kinderen.

In dit proefschrift worden de epidemiologie, risicofactoren, effecten op de hersenen en het gehoor en de ontwikkeling van een groot cohort prematuur geboren kinderen met een postnataal verworven CMV infectie beschreven.

Epidemiologie en risicofactoren van een postnataal verworven CMV infectie

In **Hoofdstuk 2** hebben we prospectief onderzoek gedaan bij prematuur geboren kinderen (met een zwangerschapsduur van minder dan 32 weken), die opgenomen waren op de NICU van het Wilhelmina Kinderziekenhuis van april 2007 tot juni 2009. Om een postnataal verworven CMV infectie vast te stellen hebben we bij alle kinderen urine verzameld bij een bezoek aan de follow-up polikliniek wanneer de kinderen de leeftijd hadden van een voldragen zwangerschap (40 weken). In deze urine werd vervolgens een CMV PCR uitgevoerd en als de urine CMV positief was werd een congenitale infectie uitgesloten met behulp van een CMV PCR van urine die direct na de geboorte was verzameld en ingevroren.

Een relatief groot aantal kinderen (112/507 [22%]) kon niet worden bestudeerd omdat ze geen urineproductie hadden tijdens het bezoek aan de polikliniek. Van de geschikte kinderen werden demografische en klinische gegevens verzameld, waaronder de data van cerebrale echografie welke wekelijks bij kinderen op de NICU wordt uitgevoerd.

Negenendertig van de 315 (12%) CMV gescreende kinderen had een postnatale CMV infectie, waarvan de meerderheid (33/39 [85%]) geen symptomen had. Met behulp van multivariabele logistische regressie analyse werden onafhankelijke risicofactoren van een postnataal verworven CMV infectie vastgesteld. Dit waren allochtone afkomst van de moeder (Odds ratio (OR) 9,6 [95% betrouwbaarheidsinterval (CI) 4,3-21,5]), zwangerschapsduur (OR 0,7 [95% CI 0,5-0,9]) en borstvoeding (OR 13,2 [95% CI 1,7-104,5]). Daarnaast was lenticulostriatale vasculopathie (LSV) die zichtbaar was op een cerebrale echo en werd uitgevoerd op de leeftijd van een voldragen zwangerschap, ook geassocieerd met een postnataal verworven CMV infectie (OR 4,1 [95% CI 1,9-8,8]).

In tegenstelling tot een eerdere studie waarin 48% van de postnataal geïnfecteerde prematuur geboren kinderen klinische symptomen van infectie had (waaronder sepsis-achtige symptomen, leverproblemen, neutropenie en thrombocytopenie), was de meerderheid van de geïnfecteerde kinderen in onze groep niet symptomatisch (85%).

Dit is in overeenstemming met een systematische review uit 2010 waarin het risico op symptomen van een postnatale CMV infectie wordt geschat op 3,7% (spreiding 0-34,5%).

Prematuur geboren kinderen kunnen een postnatale CMV infectie krijgen voordat ze de leeftijd van een voldragen zwangerschap bereiken en daarom is een dergelijke infectie mogelijk vergelijkbaar met een congenitale CMV infectie in het derde trimester van de zwangerschap. Omdat daarnaast bekend is dat CMV cellen van het zenuwstelsel kan infecteren, hebben we In dit hoofdstuk beeldvorming beschreven van alle kinderen in het cohort. De kinderen met een postnatale CMV infectie hadden een groter risico om LSV te ontwikkelen in vergelijking met niet geïnfecteerde kinderen. In een eerdere studie met congenitaal geïnfecteerde kinderen werd gesuggereerd dat er een relatie is tussen LSV en perceptief gehoorverlies. Echter, bij kinderen met een postnatale CMV infectie is de relatie tussen LSV en (neurologische) ontwikkeling en gehoor nog niet vastgesteld. Deze bevindingen waren aanleiding voor verder onderzoek op het gebied van cerebrale beeldvorming, het gehoor en de neurologische ontwikkeling van postnataal geïnfecteerde prematuur geboren kinderen. Dit onderzoek is beschreven in respectievelijk hoofdstuk 6, 7 en 8.

CMV overdracht van moeder op kind

Het risico van CMV overdracht van de moeder naar haar prematuur geboren kind varieert in verschillende studies tussen de 6 en 59%. De factoren die van invloed zijn op de CMV overdracht van moeder op kind zijn nog onvoldoende onderzocht. Eerder is gesuggereerd dat een grote hoeveelheid maternale anti-CMV antistoffen (anti-CMV IgG) geassocieerd is met een groter risico op CMV overdracht van moeder op kind.

Om dit verder te onderzoeken hebben we in **Hoofdstuk 3** de hoeveelheid maternale anti-CMV IgG onderzocht in het bloed van 79 moeders en hun 94 kinderen. Het bloed werd afgenomen binnen drie dagen na de geboorte. Negenendertig van de 94 (41%) onderzochte kinderen kregen een postnatale CMV infectie. We hebben geen significante relatie kunnen vaststellen tussen absolute hoeveelheden anti-CMV IgG van moeders of kinderen en het risico op CMV infectie van moeder op kind. Echter, een lagere anti-CMV IgG kind-moeder ratio (de hoeveelheid anti-CMV IgG van het kind gedeeld door de hoeveelheid anti-CMV IgG van de moeder) was wel geassocieerd met een grotere kans op CMV overdracht, waarbij kinderen met een lagere ratio een hogere kans hadden op een CMV infectie.

Een hoge anti-CMV IgG titer van de moeder zou veroorzaakt kunnen worden door

recente en/of frequente CMV reactivering of een infectie met een andere CMV stam. Waarschijnlijk kunnen met name deze moeders CMV doorgeven aan kinderen die een lage anti-CMV IgG titer hebben. Kinderen die zijn geboren bij een korte zwangerschapsduur en kinderen waarbij het transport van anti-CMV IgG over de placenta verstoord is zouden daarom een groter risico kunnen hebben op een postnatale CMV infectie. Omdat slechts één kind een symptomatische postnatale CMV infectie had, was het niet mogelijk om de anti-CMV IgG titers en ratio van symptomatische en niet symptomatische kinderen te vergelijken.

Ernst van de ziekte in relatie met CMV load en genotype

In tegenstelling tot de voornamelijk niet symptomatische postnatale CMV infectie bij prematuur geboren kinderen, presenteren voldragen geboren kinderen met een congenitale CMV infectie die opgenomen worden op de NICU zich vaak met ernstige symptomen. Meerdere studies bij congenitaal geïnfecteerde kinderen hebben aangetoond dat er een relatie is tussen de hoeveelheid CMV kopieën in de urine en het bloed (CMV load) en ernst van de ziekte, waarbij symptomatische kinderen een hogere CMV load hadden dan niet symptomatische kinderen. Ook de latere ontwikkeling van perceptief gehoorsverlies in kinderen met een congenitale CMV infectie was geassocieerd met een hogere CMV load. In **Hoofdstuk 4** hebben we de CMV load in de urine van 45 kinderen met een postnatale CMV infectie vergeleken met de CMV load in urine van 17 kinderen met een congenitale CMV infectie. Hierbij was de CMV load van kinderen met een congenitale CMV infectie significant hoger dan van kinderen met een postnatale CMV infectie.

In eerdere studies is ook gesuggereerd dat de ernst van de ziekte bij kinderen met een congenitale CMV infectie afhankelijk is van het CMV genotype. Daarom hebben we in **Hoofdstuk 5** in 58 prematuur geboren kinderen met een postnatale CMV infectie het CMV UL55 en UL144 genotype bepaald en de verdeling hiervan vergeleken met de genotype verdeling van 13 kinderen met een congenitale CMV infectie. Hieruit bleek dat de verdeling van verschillende genotypen niet verschillend was, terwijl de ziektelast bij congenitaal geïnfecteerde kinderen gemiddeld hoger was. Dit kan suggereren dat de ernst van de ziekte niet afhankelijk is van het genotype. Een analyse van CMV load in vergelijking met het genotype toonde geen significante verschillen aan tussen de genotypen.

Cerebrale beeldvorming

Er zijn geen eerdere studies over cerebrale beeldvorming bij prematuur geboren

kinderen met een postnatale CMV infectie. In hoofdstuk 2 hebben we met behulp van cerebrale echografie de associatie tussen LSV en een postnatale CMV infectie onderzocht. Naast cerebrale echografie wordt een MRI-scan van de hersenen ook gebruikt om witte stofschade te bepalen bij prematuur geboren kinderen. Cerebrale echografie is vooral nuttig bij ziektes die cysten in de hersenen veroorzaken en een MRI-scan kan meer subtiele witte stofschade zichtbaar maken. In eerdere studies bij prematuur geboren kinderen is het verband gelegd tussen witte stofschade bij een voldragen leeftijd (40 weken) en een ongunstige neurologische ontwikkeling. In hoofdstuk 4 en 5 hebben we MRI-scans geëvalueerd van prematuur geboren kinderen met een postnatale CMV infectie, waarbij geen afwijkingen werden gevonden. Daarom hebben we in **Hoofdstuk 6** 'diffusion tensor imaging' (DTI) gebruikt waarbij de snelheid en richting van de diffusie van water in de hersenen bepaald wordt om kleine structurele witte stofveranderingen aan te tonen. De resultaten hiervan worden uitgedrukt in verschillende parameters, waaronder gemiddelde diffusie (MD) en fractionele anisotropie (FA). Deze parameters hebben we bepaald in drie witte stofgebieden (frontaal, pariëtaal en occipitaal) van 21 postnataal CMV geïnfecteerde prematuur geboren kinderen en 61 niet geïnfecteerde prematuur geboren kinderen. Het bleek dat de FA van kinderen met een postnatale CMV infectie in de occipitale witte stof significant lager was dan bij niet geïnfecteerde kinderen. Omdat de MD in dit gebied (en in de andere gebieden) niet verschillend was tussen beide groepen, kan dit er op wijzen dat er kleine structurele witte stofveranderingen zijn in de occipitale witte stof bij kinderen met een postnatale CMV infectie. Om te kijken of deze veranderingen ook relevant waren voor de ontwikkeling van deze kinderen hebben we de ontwikkeling getest bij een voor de prematuriteit gecorrigeerde leeftijd van 16 maanden. In 19/21 (90%) kinderen met een postnatale CMV infectie en 60/61 (98%) niet geïnfecteerde kinderen kon met behulp van de Griffiths Mental Developmental Scales (GMDS) de ontwikkeling worden bepaald. Er werden geen verschillen tussen postnataal geïnfecteerde en niet geïnfecteerde groepen vastgesteld. Het lijkt daarom dat kleine structurele witte stofveranderingen in de occipitale witte stof bij kinderen met een postnatale CMV infectie niet geassocieerd zijn met een ongunstige ontwikkeling bij 16 maanden.

Langetermijneffecten van een postnatale CMV infectie

Er zijn weinig studies over de langetermijneffecten van een postnatale CMV infectie in prematuur geboren kinderen. Deze langetermijneffecten kunnen belangrijk zijn omdat sommige congenitaal geïnfecteerde kinderen een ernstige ontwikkelingsstoornis en perceptief gehoorsverlies ontwikkelen.

Onderzoek naar het gehoor van prematuur geboren kinderen met een postnatale CMV infectie is schaars en beperkt door kleine groepen. In **Hoofdstuk 7** hebben we metingen van hersenstam geëvokeerde potentialen (ABR) gebruikt om het gehoor van 64 prematuur geboren kinderen met postnatale CMV infectie te meten in het eerste levensjaar en het gehoor van 18 kinderen in het tweede levensjaar. In dit grote cohort van kinderen heeft geen enkel kind perceptief gehoorsverlies ontwikkeld. Omdat de meeste ouders in het tweede levensjaar overtuigd waren van het goede gehoor van hun kind zijn niet alle kinderen getest. Daarom hebben we de taalontwikkelingschaal van de GMDS gebruikt bij een gecorrigeerde leeftijd van 16 maanden om het gehoor van 14 kinderen waarvan het gehoor was getest in het tweede levensjaar, te vergelijken met 44 kinderen waarvan het gehoor niet was getest. Er waren geen verschillen wat betreft ontwikkeling, noch wat betreft taalontwikkeling, wat erop kan wijzen dat er geen ernstig perceptief gehoorsverlies was in de 44 niet geteste kinderen.

De ontwikkeling van prematuur geboren kinderen met een postnatale CMV infectie is onvoldoende bestudeerd. Er zijn slechts twee opeenvolgende case-control studies die een normale cognitieve en motorische ontwikkeling beschrijven in 20 prematuur geboren kinderen met en zonder een postnatale CMV infectie. Volgend op onze studies beschreven in hoofdstuk 2, 6 en 7 hebben we in **Hoofdstuk 8** met behulp van twee ontwikkelingstesten vanaf 2009 propectief de ontwikkeling bepaald van alle kinderen in het cohort. Bij een gecorrigeerde leeftijd van 16 maanden werd de GMDS test uitgevoerd in 390 prematuur geboren kinderen, waarvan er 57 (15%) een postnatale CMV infectie hadden verworven voor de voldragen leeftijd. Vervolgens werd op de gecorrigeerde leeftijd van 24 maanden een Bayley Scales of Infant and Toddler Development (BSID-III) test afgenomen in 328 kinderen, waarvan er 53 (16%) een postnatale CMV infectie hadden. Verder werd er in 12 CMV positieve kinderen en 55 CMV negatieve kinderen een GMDS test afgenomen in plaats van de BSID-III test. De kinderen met een postnatale CMV infectie waren niet behandeld met antivirale medicatie of anti-CMV antilichamen.

Het was opvallend dat op een leeftijd van 16 maanden CMV geïnfecteerde kinderen een betere grove motorische ontwikkeling hadden dan niet geïnfecteerde kinderen. Er waren

geen verschillen in algemene ontwikkeling. Met behulp van de meer gedetailleerde BSID-III op een leeftijd van 24 maanden werden geen verschillen gevonden. Echter, kinderen met een postnatale CMV infectie begonnen significant vroeger met los lopen in vergelijking met niet geinfecteerde kinderen. Multivariabele regressie analyse toonde dat etniciteit van de moeder en niet de postnatale CMV infectie het meest waarschijnlijk verantwoordelijk was voor dit verschil.

Net zoals in hoofdstuk 2, werd er een verhoogde incidentie van LSV gezien bij de kinderen met een postnatale CMV infectie. De ontwikkeling van LSV in de eerste levensmaanden was niet geassocieerd met een vertraagde ontwikkeling.

Omdat slechts vier kinderen een symptomatische infectie hadden, en de ontwikkeling in slechts één symptomatisch kind getest werd, was het niet mogelijk om te onderzoeken of kinderen met een symptomatische postnatale CMV infectie een slechtere ontwikkeling hebben dan kinderen zonder symptomen.

Preventie en behandeling

In hoofdstuk 2, 7 en 8 hebben we aangetoond dat een postnatale CMV infectie in prematuur geboren kinderen meestal asymptomatisch is en waarschijnlijk niet resulteert in gehoorsproblemen of een vertraagde ontwikkeling op een gecorrigeerde leeftijd van twee jaar. Omdat de nadelen van preventieve maatregelen, zoals het invriezen, pasteuriseren of zelfs onthouden van borstvoeding hoogstwaarschijnlijk niet opwegen tegen de risico's van een postnatale CMV infectie, lijken deze maatregelen niet noodzakelijk. Geen van de kinderen met een postnatale CMV infectie die werden bestudeerd in dit proefschrift hebben antivirale medicatie of intraveneuze anti-CMV antilichamen gekregen. In alle kinderen met een symptomatische postnatale CMV infectie, waren de symptomen voorbijgaand. De veiligheid en effectiviteit van antivirale medicatie (Ganciclovir® en Valganciclovir®) is niet vastgesteld in prematuur geboren kinderen. Intraveneuze toediening van anti-CMV antilichamen kan nuttig zijn bij kinderen met symptomen, maar de effectiviteit is ook niet onderzocht. Behandeling van prematuur geboren kinderen met een postnatale CMV infectie zou alleen moeten worden overwogen bij levensbedreigende symptomen.

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Dankwoord

"Het leven is wat je gebeurt terwijl je andere plannen maakt"

Acda & De Munnik – Laat me slapen

Toen ik midden 2009 ging praten met **dr. Malgosia Verboon-Maciolek** over een wetenschappelijke stage op de NICU met als onderwerp postnatale cytomegalovirus infecties bij prematuur geboren kinderen, heb ik geen moment gedacht dat er vier jaar later een proefschrift met mijn naam erop over datzelfde onderwerp in uw handen zou liggen. De keuzes van een aantal mensen hebben ertoe geleid dat ik mij de afgelopen jaren in heb kunnen zetten voor dit belangrijke onderzoek bij deze zeer kwetsbare kinderen. Nog veel meer mensen hebben ervoor gezorgd dat het onderzoek een succes is geworden en uiteindelijk heeft geresulteerd in mijn promotie en dit proefschrift. Ik wil hiervoor een ieder die hieraan heeft bijgedragen hartelijk bedanken en een aantal mensen bij naam noemen.

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Drs. Niek van der Aa, Drs. Johanneke Harteman, Drs. Karina Kersbergen, Dr. Britt van Kooij, Drs. Margareta Vogelaar-Brouwer, Drs. Hilde Bonestroo, beste mede-promovendi. Ik wil jullie hartelijk bedanken voor alle hulp en gezelligheid. Ik wens jullie ook heel veel succes met het afronden van jullie onderzoek, in zoverre dat nog niet afgerond is.

Niek en Britt, ik blijf het jammer vinden dat we die 1000 dollar in Vancouver niet hebben gewonnen. En Niek, ik zal me de terugvlucht naar Chicago de ochtend daarna nog lang heugen. Succes in Amersfoort!

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"Life is too important to take it seriously"

Oscar Wilde

Tjeerd & Danielle, Lars & Noortje, Martin & Paula, Nolan & Marion, Bart, Stefan & Laura. *Nazomer 2003, Willy's Winkeltje.* Ik moest boeken kopen voor het tweede jaar biomedische wetenschappen omdat ik opnieuw was uitgeloot voor geneeskunde. Een vriendelijke geblondeerde kerel gaf me wat tips over de aan te schaffen boeken en in de pauze van college kreeg ik koffie van een oudere kerel die in tegenstelling tot de gemiddelde BMW-student een echte witte pas had. Flink wat gezamenlijke studieopdrachten, practica en vervolgens stapavonden, bordjes pasta-pepersaus, PS3 games, afleveringen How I Met Your Mother, weekendjes, vakanties, landen, auto's (wijlen de Citroen BX en de Renault Espace V6), aanhang en zelfs jaren van samenwonen later, voel ik me gezegend met zo'n fantastische vriendengroep.

Bro's, het is weer tijd voor PPS!

Ruben, Arja, Nienke, Francine en Floor. Ik wist zeker dat ik na ons zilveren jaar, het leukste jaar in mijn studietijd, gouden vrienden had gemaakt. Nu iedereen is afgestudeerd en de rat race naar een opleidingsplek of leuke baan is begonnen, waardeer ik het des te

meer dat we elkaar nog zo regelmatig zien. Van belangrijke beslissingen over werk, wonen en leven tot een goeie wijn drinken en naar een concert gaan, ik weet dat ik altijd bij jullie terecht kan (en uiteraard visa versa).

Maartje. Biertjes tappen op de Hamburgerstraat doen we helaas al een poosje niet meer, maar elkaar op de hoogte houden van de dagelijkse beslommeringen, het ophangen van rolgordijnen en een feestje op z'n tijd gelukkig wel. Ik geef toe dat de frequentie op het moment suboptimaal is, maar daar wordt aan gewerkt!

Maarten, Wilmar, Roos & Sicco, Renée, Rutger. Of het nou op een verjaardag, festival of gewoon thuis is; of het nou over muziek, wijn, auto's of politiek gaat; of jullie nou van het op-1-na leukste bestuur van Sams zijn, geneeskunde hebben gestudeerd of iets met cultuur doen, het maakt me niet uit, het is leuk om bij jullie te zijn.

Bart en Gerbrand. Eetdate, herkansing 3.0, ik ben er bij, echt!

Leden van het Medisch Dispuut Equivoque, het leukste dispuut van Utrecht. Als het eerste oud-lid, honorair, reünist (volgens mij hebben we de naam nog niet bedacht) van EQQ ben ik trots dat ons dispuut bloeit en hoop ik dat ik jullie snel weer tref.

Bert. Van een raket in elkaar knutselen tot promoveren, het kan verkeren. Al 27 jaar giet 't zoas 't giet, en dat vind ik machtig mooi.

Lieve **Clemens & Carla, Nicole & Rick, Fleur.** Al een aantal jaar vind ik het erg gezellig met jullie. Met z'n allen eten, een boottochtje maken op de Loosdrechtse plassen of skiën in Vallandry: er wordt altijd gelachen en daar geniet ik van. Ondanks dat ik jullie de afgelopen maanden niet erg veel kon zien, heb ik jullie steun bij het afronden van mijn proefschrift erg gewaardeerd.

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Ik vind het ontzettend fijn dat ik naast jullie serieuze adviezen ook zeer regelmatig kan genieten van jullie humor, interesse in politiek en beestjes haken, liefde voor koken, wijn, muziek en warme landen en een leuk huis met dito veestapel. Of we nou in Hengelo, Utrecht, Frankrijk of Montalcino afspreken, het voelt altijd als thuis.

Lieve **Francine**, allerliefste, ben je toch als laatste in dit laatste hoofdstuk beland. Geheel onterecht natuurlijk, want eigenlijk zou je centraal moeten staan. Sinds iets meer dan drie jaar heb ik het geluk om mijn leven met jou te mogen delen en dat doe ik elke dag met ongelofelijk veel plezier. Ik vind het onvoorstelbaar hoeveel steun je me hebt gegeven om dit proefschrift af te kunnen maken. Je hebt de inhoud (en de omslag) van dit proefschrift kritisch bekeken, gecorrigeerd en op meerdere momenten aangevuld. Daarnaast heb je ervoor gezorgd dat ik me de afgelopen weken, naast mijn werk in de kliniek volledig kon richten op het voltooien van dit boekje, hoe ongezellig dat vaak ook was. Je schoof regelmatig ongemerkt een bordje eten op schoot als ik 's avonds door moest werken. Ondertussen werk je flink door aan je eigen promotie-onderzoek, waar je gelukkig ook nog een proefschrift van gaat maken, want dan kan ik tenminste nog iets terug doen. Ondanks dat dit boekje nu klaar is ga ik met jou nog heel veel hoofdstukken schrijven. Het is heerlijk om met jou te dromen over alles wat we allemaal gaan doen en ik verheug me dan ook op het volgende hoofdstuk!

Curriculum vitae

Joppe Nijman was born on the 5th of October 1983 in Emmen, The Netherlands. He grew up in Emmen and Nieuw-Amsterdam, a small village next to Emmen. It was there he went to primary school, C.B.S. de Bron. In 2002, he finished secondary school (Voortgezet Wetenschappelijk Onderwijs) with the direction Nature & Health and graduated at the Hondsrug College, Emmen. Subsequently, he moved to Utrecht, and in September 2002 started studying Biology at the Utrecht University. After finishing his first year of Biology, he enrolled the second year of Biomedical Sciences, also at the University of Utrecht. In 2005, he was admitted to the Selective Utrecht Medical Master (SUMMA), a shortened medicine study (4 years) at the University Medical Center Utrecht. During his medicine study, in 2007-2008, he was treasurer of the board of the Faculty of Medicine Students Association, MSFU "Sams".

In 2009, after completing his internships at Gelre Ziekenhuis in Apeldoorn, The Netherlands, he started working on his research project on postnatal cytomegalovirus infections in preterm infants, under supervision of dr. Malgosia Verboon-Maciolek at the Neonatal Intensive Care Unit (NICU) of the Wilhelmina's Childrens Hospital (WKZ) in Utrecht, The Netherlands. This research project resulted in current thesis. In January 2011 he finished medical school and subsequently, worked as a resident at the Pediatric Intensive Care Unit of the WKZ.

From August 2011 until November 2012 he finished this thesis at the NICU of the WKZ. Furthermore, he completed the first phase of the Effective Perinatal Care in Europe (EPICE) study in the Utrecht region, under supervision of prof. dr. Louis Kollee and dr. Corine Koopman-Esseboom. He started in November 2012 as a resident in pediatrics in the WKZ. In August 2013 he will start with his speciality registrar in pediatrics at the Gelderse Vallei hospital in Ede, The Netherlands.

Joppe lives together with Francine van Erp in Utrecht.

Curriculum vitae

Joppe Nijman werd geboren op 5 oktober 1983 in Emmen, Nederland. Hij groeide vervolgens op in Emmen en Nieuw-Amsterdam, een dorpje bij Emmen. Daar volgde hij het basisonderwijs op basisschool C.B.S. de Bron. In 2002 haalde hij zijn diploma van het Voortgezet Wetenschappelijk Onderwijs (VWO) met het profiel Natuur & Gezondheid bij het Hondsrug College te Emmen. Vervolgens verhuisde hij naar Utrecht om in september 2002 Biologie te gaan studeren aan de Universiteit Utrecht. Na het eerste jaar van deze studie kon hij in 2003 instromen in het tweede jaar van Biomedische Wetenschappen, ook aan de Universiteit Utrecht. In 2005 werd hij toegelaten tot de verkorte Geneeskunde opleiding, Selective Utrecht Medical Master (SUMMA, 4 jaar) aan de Universiteit Utrecht / Universitair Medisch Centrum Utrecht. Na de eerste twee jaar te hebben afgerond heeft hij in het academisch jaar 2007-2008 fulltime als penningmeester in het bestuur van de Medische Studenten Faculteitsvereniging "Sams" gezeten.

In 2009, na het afronden van zijn co-schappen in het Gelre ziekenhuis te Apeldoorn, is hij onder begeleiding van dr. Malgosia Verboon-Maciolek op de NICU van het Wilhelmina Kinderziekenhuis (WKZ) te Utrecht begonnen aan het onderzoeksproject over postnataal verworven cytomegalovirus infecties bij prematuur geboren kinderen. Dit was het begin van het traject dat uiteindelijk zou resulteren in deze dissertatie. In januari 2011 ontving hij zijn artsenbul om vervolgens een half jaar op de Pediatrische Intensive Care Unit van het WKZ te werken als arts-assistent kindergeneeskunde niet in opleiding. Van augustus 2011 tot november 2012 werkte hij verder aan zijn promotie-onderzoek op de NICU van het WKZ en heeft hij als arts-onderzoeker de eerste (klinische) fase van het Effective Perinatal Care in Europe (EPICE) onderzoek voor de regio Utrecht voltooid. Vervolgens is hij in november 2012 in het WKZ te Utrecht begonnen als arts-assistent kindergeneeskunde niet in opleiding. In augustus 2013 begint hij aan de opleiding tot kinderarts in Ziekenhuis Gelderse Vallei te Ede.

Joppe woont samen met Francine van Erp in Utrecht.

Notes