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Early Postnatal Infection with Human Cytomegalovirus Has Long-Term Consequences on Brain Structure of Former Preterm Born Children

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List of Abbreviations

ADHD	attention deficit hyperactivity disorder
AMAP	adaptive maximum a posterior
ANOVA	analysis of variances
BPD	bronchopulmonary dysplasia
BrainAGE	brain age gap estimation maturation
CAT12	computational anatomy toolbox version 12
CSF	cerebrospinal fluid
CSFV	volume of cerebrospinal fluid
DARTEL	fast diffeomorphic image registration algorithm
dMRI	diffusion-weighted magnetic resonance imaging
EHI	Edinburgh Handedness Inventory
FG	Frühgeborene (German for preterm born participants)
fMRI	functional magnetic resonance imaging
FT	full-term born participants
FWE	family-wise error rate
FWHM	full width half maximum
GLM	general linear model
GM	grey matter
GMV	grey matter volume
HAWIK IV	Hamburg Wechsler Intelligenztest für Kinder IV
hCMV	human cytomegalovirus
hCMV+	seropositive for hCMV
hCMV-	seronegative for hCMV
HMRF	hidden Markov random field
ICH	intracranial hemorrhage
IQ	intelligence quotient
IQR	image quality rating
IVH	intraventricular hemorrhage
ME	maternal education
MR	magnetic resonance
MRF	Markov random field
MRI	magnetic resonance imaging
NEC	necrotizing enterocolitis
NICU	neonatal intensive care unit
PT	preterm born participants
PT_{hCMV-}	preterm born participants without early postnatal hCMV infection
PT_{hCMV+}	preterm born participants with early postnatal hCMV infection
PVL	periventricular leukomalacia
RF	radiofrequency impulse
RG	Reifgeborene (German for full-term born paritcipants)
RP	retinopathy
RDS	respiratory distress syndrome
SEM	standard error of the mean
SPH	spherical harmonic
SPM12	statistical parametric mapping version 12 package
TE	echo time
TFCE	threshold-free cluster enhancement
TIV	total intracranial volume
TOM	template-o-matic
TR	repetition time
VBM	voxel-based morphometry
WM	white matter
WMV	white matter volume

1 Introduction

1.1 Preterm Birth

1.1.1 Definition and Epidemiology

Preterm birth is defined as birth before 37 weeks of gestation. Preterm births are further classified into extremely preterm born babies, born before 28 weeks of gestation, very preterm born babies (28 to before 32 weeks), moderate preterm born babies (32 to before 34 weeks) and late preterm born babies (34 to before 36 weeks). In total, 14.9 million babies were born preterm in 2010, corresponding to 11.1% of livebirth; and global numbers of preterm born babies are rising. The highest rates are found in Southeastern and South Asia and Sub-Saharan Africa, peaking at 18% in Malawi, with the lowest rates being recorded in Northern Europe. (Blencowe et al., 2013, Blencowe et al., 2012) In Europe, preterm birth rates ranged from 5.5-11.1% of livebirths in 2008. (Zeitlin et al., 2013) The latest numbers in Germany report a rate of 8.64% in 2016, of which 0.59% were in the high-risk extremely preterm born children category and 0.9% were very preterm born children (Schill et al., 2017). While worldwide numbers of preterm births are increasing, some countries in Europe and the USA have reported declining numbers of preterm births. (Zeitlin et al., 2013, Schoen et al., 2015) In Germany, numbers have been stable over the past years. (Schleußner, 2013)

1.1.2 Causes

Preterm births are classified into spontaneous and indicated preterm deliveries. Preterm delivery is indicated when fetal or maternal life is at risk. Maternal indication includes hypertensive disorders, such as preeclampsia; fetal indication includes suspected fetal growth restriction and placental abruption. Spontaneous preterm birth arises in case of preterm labor and premature rupture of membranes. Preterm labor is the most common cause of preterm birth, followed by premature rupture of membranes. (Delorme et al., 2016)

Various factors have been found to increase risk of spontaneous preterm delivery. Previous preterm delivery is considered the most important risk factor.

(Koullali et al., 2016, Kazemier et al., 2014) Preceding cervical surgery (Jakobsson et al., 2007), genetic predisposition (Monangi et al., 2015), lower maternal education (Ruiz et al., 2015), low socio-economic status (Smith et al., 2007), African and Afro-Caribbean ethnicity (Goldenberg et al., 2008), high-strain jobs (Brett et al., 1997), maternal stress (Kajeepeta et al., 2014), smoking (McCowan et al., 2009), underweight and obesity (Khashan and Kenny, 2009, Shaw et al., 2014), and periodontal diseases (Offenbacher et al., 1996) are further maternal risk factors. Environmental characteristics like heat waves, air pollution (Schifano et al., 2013) and low socio-economic status of a mother's neighborhood (Wallace et al., 2016, Smith et al., 2007) may also increase risk of preterm delivery. Pregnancy characteristics associated with preterm birth are multiple pregnancy (Blondel et al., 2006), short inter-pregnancy interval (Shachar et al., 2016), intrauterine infections (Lee et al., 2008, Goldenberg et al., 2000), bacterial vaginosis (Hillier et al., 1995), short cervical length in mid-pregnancy (Iams et al., 1996) and the use of assisted reproductive technologies (Dunietz et al., 2015). In summary, reasons for preterm birth are diverse and, in many cases, impossible to avoid. Therefore and although numbers of preterm deliveries have been declining (Schoen et al., 2015), preterm birth will continue to pose substantial medical and societal challenges.

1.1.3 Short-Term Consequences

Preterm birth is the most common cause of neonatal death. (Frey and Klebanoff, 2016) In live-born preterm born individuals, mortality rate is 5% on average. Mortality rate decreases drastically with increasing gestational age of preterm babies. At 24 weeks of gestation, more than 70% of preterm babies die; at 25 weeks, mortality rate has already decreased below 40%. The mortality rate at 29 weeks of gestation, or older, is below 5% (Delorme et al., 2016). In Germany, the mortality rate of babies born before 32 weeks of gestation in 2016 was 4.15% (Schill et al., 2017).

In neonatal intensive care units (NICU), preterm born infants may develop several serious complications. One of the most common complications is respiratory distress syndrome (RDS), which requires artificial invasive or non-invasive ventilation (Hermansen and Lorah, 2007). Artificial ventilation and further invasive

treatment can result in bronchopulmonary dysplasia (BPD), defined as requiring supplementary oxygen at term (Jobe, 2011, Poindexter et al., 2015). Due to immature liver metabolism and consequently increased levels of bilirubin, preterm born infants often develop hyperbilirubinemia (Stevenson et al., 2001), requiring phototherapy (Maisels and Watchko, 2014) or blood exchanges. Another common complication is necrotizing enterocolitis (NEC), an inflammatory intestinal disease often accompanied by bowel perforation which may require surgical intervention (Claud and Walker, 2001, Heida et al., 2017). Preterm babies are also at risk of developing retinopathy of prematurity (ROP), resulting in visual impairment and, in severe cases, blindness (Sapieha et al., 2010). Neurologic complications are intraventricular hemorrhage (IVH) which are graded from mild (grade I) to severe (grade IV) and occur mostly due to vulnerable vessels in the periventricular germinal matrix (Ballabh, 2010). Periventricular leukomalacia (PVL) describes white matter damage due to ischemia and ensuing inflammatory events (Volpe, 2009).

Among very preterm born babies born in 2016 in Germany, the rate of BPD was 6.28%, the rate of higher-grade IVH was 3.75%, the rate of higher-grade ROP was 3.36%, the rate of PVL was 1.38%, and the rate of NEC requiring surgical intervention was 1.16% (Schill et al., 2017). Similar to mortality, morbidity also increases with decreasing gestational age (Stoll et al., 2010, Scheuchenegger et al., 2014). Data on preterm born babies born <29 weeks of gestation, published by the neonatal research network of the USA, reported strikingly higher rates of morbidity; especially of BPD (68%) and ROP (59%) in the more preterm born babies (Stoll et al., 2010).

1.1.4 Long-Term Consequences

Preterm birth has a substantial impact on the overall burden of disease as the surviving babies can suffer from chronic ailments for a very long period of time (Harrison and Goldenberg, 2016). Therefore, long-term consequences of preterm birth have been investigated in a large number of studies.

First, preterm birth has long-term physical consequences. Preterm born people with, but also without, a history of overt BPD were at a higher risk of suffering from chronic pulmonary problems. Furthermore, glomerular filtration rate of

school-aged preterm born children was decreased, possibly indicating long-term kidney impairment. Former preterm born adults showed a higher risk of suffering from hypertension, type 2 diabetes, and obstructive sleep apnea syndrome. Moreover, risk of thromboembolic events during childhood was significantly increased. (Raju et al., 2017)

Second, preterm birth has long-term neurologic and neuropsychological sequelae. Some former preterm born children develop severe motor impairment, like cerebral palsy, affecting 3.5% of children born with a birth weight of <1500g (Sellier et al., 2016). Severe cognitive deficits, affecting 4% of extremely preterm born children (Moster et al., 2008), and severe visual or hearing impairment, affecting 1% of extremely preterm born children are further overt manifestations (Johnson and Marlow, 2017). In contrast, preterm born individuals with a relatively good outcome showed more subtle deficits. At school-age, preterm born children show a lower intelligence quotient (IQ) (Bhutta et al., 2002, Raju et al., 2017). Cognitive deficits of very preterm born children persisted throughout adolescence into adulthood (Eryigit Madzwamuse et al., 2015). Preterm born children on average scored one standard deviation below full-term born individuals (Raju et al., 2017). And yet again, the lower the birth weight and the gestational age, the lower children scored at intelligence tests (Bhutta et al., 2002). Although high hopes were set onto improvements in long-term outcome of preterm born infants, and although overt manifestations such as cerebral palsy are declining (Sellier et al., 2016) due to improved neonatal care, recent studies still reported persisting cognitive impairment in very preterm born children and extremely preterm born children. (Hutchinson et al., 2013, Anderson, 2014)

Specific neurocognitive domains are also quantifiably affected. School-aged very preterm born children and extremely preterm born children showed language delays usually persisting into adulthood (Vohr, 2014), higher rates of reading disabilities (Bowen et al., 2002, Hutchinson et al., 2013, Aarnoudse-Moens et al., 2011), and even greater difficulties in arithmetic tasks (Aarnoudse-Moens et al., 2011, Hutchinson et al., 2013, Johnson and Marlow, 2017). More preterm born children took part in special education classes or required additional learning support (Bowen et al., 2002, Wocadlo and Rieger, 2007, Spencer et al., 2008).

Consequently, fewer preterm born children completed basic school (Mathiasen et al., 2010) and fewer preterm born people attained higher educational levels. With decreasing gestational age, the ultimate likelihood of earning a low income increased, although the rate of unemployment was not associated with gestational age. (Moster et al., 2008)

Finally, there is a higher probability for behavioral issues. For example, 3-year-old very preterm born children already showed behavioral problems, including hyperactivity-inattention, peer problems, and conduct problems (Delobel-Ayoub et al., 2006). At school-age, very preterm born children showed a three-fold risk for any psychiatric disease, a two-fold rate of ADHD (attention deficit hyperactivity disorder), and higher than average rates in anxiety disorders and autism spectrum disorders (Treyvaud et al., 2013). Many, though not all studies, reported poorer social skills in childhood and adolescence (Ritchie et al., 2015). Parents and teachers of extremely preterm born children likewise observed higher rates of inattention and problems in social functioning (Scott et al., 2012).

These issues are carried on past childhood and adolescence. Preterm born adults reported poorer health and more psychological problems (Husby et al., 2016). Incidence of psychiatric disorders was increased in preterm compared to full-term born adults and increased with decreasing gestational age (Lindstrom et al., 2009). Interestingly, preterm born adults reported fewer depressive symptoms and higher health-related quality of life than their full-term born peers (Dalziel et al., 2007).

In summary and as described above, many studies report a high risk of physical, cognitive, psychological, and social long-term sequelae in former preterm born children and adults. These reports indicate that research on the pre-, neonatal, and later risk factors associated with preterm birth is necessary and relevant for our society.

1.2 Magnetic Resonance Imaging and Brain Morphometry

1.2.1 Physical Principles of Magnetic Resonance Imaging

Magnetic resonance imaging (MRI) is based on the principle of nuclear magnetic resonance. The concept posits that electrons, neutrons, and protons re-emit

electromagnetic waves, following stimulation by an electromagnetic pulse. Electrons, neutrons, and protons exhibit a so-called spin, which has features of a magnetic dipole. Pairs of spins nullify each other; therefore, only nuclei with an uneven number of nucleons exhibit a net spin. For instance, hydrogen nuclei, consisting of one proton only, emit a net spin. Since body tissues feature high hydrogen concentrations, human MRI usually measures hydrogen spins. If not externally stimulated, spins will arrange in a random direction. If spins are influenced by an external magnetic field, however, some will arrange accordingly. Randomly arranged spins within a tissue or object nullify each other and no electromagnetic waves are emitted. In case of external magnetic stimulation, a certain amount of spins arranges itself in line with the magnetic field lines. After being excited by an appropriately-tuned radiofrequency (RF) impulse, the resulting cumulative signal can then be measured. By spatially encoding the magnetic signal, using weaker (so-called gradient) magnetic fields, the distribution of nuclei can be visualized in a magnetic resonance (MR) image.

T1 describes the time needed to reestablish the magnetization vector of spin arrangement to the static magnetic field. Therefore, T1 depends on the surrounding magnetic noise and the spin's emission and absorption of energy of surrounding particles, also described as spin-lattice interaction. Tissues with short relaxation times (fat) are hyperintense and tissues with longer relaxation times (fluids) are hypointense. Grey and white tissue of the brain differs strongly in T1; thus, brain tissue shows high contrast in T1-wheighted images.

T2 describes decay of the magnetization vector of the spin arrangement to the RF. Similarly to T1, T2 depends on spin-lattice interaction, which describes emission and absorption of energy to surrounding particles. Further, T2 is influenced by spin-spin interaction which depends on the homogeneity of the surrounding magnetic noise field. Tissues with long relaxation times (fluids) are still hyperintense, whereas tissues with shorter relaxation times (fat) are already intermediate.

Repetition time (TR) of RF depends on relaxation time. High MR signals in T1 can only be measured after sufficient relaxation time. The lower TR is, the lower T1 MR signals are. Therefore, TR determines contribution of T1 and T2 on MR

images. This weighting is also influenced by the time one waits until acquiring the signal (time to echo, TE).

Spatial encoding is obtained by overlaying a weak, oscillating, longitudinal magnetic field. This slightly changes the overall magnetic field so that effectively only one plane of spins is stimulated by the strong RF. Hence, plane selection enables assessment of spin position in, for example, the z-direction. Position of spins in x-plane is assessed by frequency encoding, since spins rotate at different Larmor frequencies. Spins further show varying phase differences following short repetitive stimulation in y-plane. Thus, position of spins in y-plane can be assessed by phase encoding. Transformation of measured signals into an image is finally achieved by Fourier transformation. (Pykett et al., 1982, Schneider and Fink, 2007)

1.2.2 Clinical and Scientific Application of MRI

MRI has become a popular imaging method for clinical and scientific applications. Contrary to conventional X-ray, computed tomography and positron emission tomography, MRI does not expose subjects to ionizing radiation and is, at present state of knowledge, not harmful for subjects in the short- and long-term, specifically including children (Holland et al., 2014). Furthermore, MRI yields superior soft tissue contrast enabling detailed inspection of muscles, tendons, and brain tissue. Consequently, MRI is an essential tool for assessing lesions or aberrations in the central nervous system.

Several advanced MR techniques exist. For example, functional MRI (fMRI) assesses neuronal activation via changing blood flow in activated brain regions, ^{1}H-MR-spectroscopy allows assessing several important metabolites, perfusion MRI is used to evaluate blood perfusion in specific brain regions, and diffusion MRI (dMRI) enables detection of cytotoxic edema (Schneider and Fink, 2007). Based on dMRI, tractography of white matter tracts in the brain is also possible (Basser et al., 2000). As none of these methods are used in the current work, they will not be discussed further.

With the help of “statistical parametric mapping” (SPM), normal and aberrant brain structure can be analyzed. Through these methods, psychiatric disorders have been associated with deviant brain structure, aiding in our understanding of

and attitude towards psychiatric disorders (Friedman and Rapoport, 2015, Fornito and Bullmore, 2015). These approaches effectively assess regional brain volume, However, high-resolution T1 images also enable further detailed analyses of brain structure. To this effect, cortical thickness, gyrification, and sulcal depth can be analyzed and be compared between groups. These methods are applied in the present paper and are therefore described in more detail in the section below (1.2.4 and 1.2.5).

1.2.3 Challenges of Performing MRI in Children

Pediatric MRI poses practical and methodical challenges. Children require calm and comforting instructions before scanning. To lie still during scanning is a demanding, and sometimes impossible, task for children. Once in the scanner, children are frequently addressed using an audio system, and, if possible, parents are allowed to stay with the children during scanning and children are entertained with short movies during structural MRI. (Wilke et al., 2003a) Children's brain structure changes rapidly during development. This is especially relevant in young children, when white matter is still unmyelinated in some areas, and grey matter features different tissue composition and water content, requiring special scanning protocols. Different tissue composition, different global proportions and different local tissue distribution in children's brains strongly call for the use of pediatric reference data in preprocessing. (Wilke and Holland, 2008b) For example, using an adult template in pediatric samples requires more deformation to be applied to the children's brains than the use of a pediatric template (Wilke et al., 2002, Yoon et al., 2009). Especially structural and functional imaging data with higher resolution may be biased when using an adult template, and consequently, using pediatric reference data is highly recommended (Wilke et al., 2008, Wilke et al., 2003b, Yoon et al., 2009, Wilke et al., 2002).

1.2.4 Voxel-Based Morphometry

Voxel-based morphometry (VBM), as the name implies, analyzes the brain's structure on the voxel level of commonly about 1 mm^3. Prior to voxel-wise analysis, images inevitably need to be preprocessed. They are registered and warped to a reference brain, to minimize individual variance, and smoothed, to

remove noise and to approach a normal (Gaussian) distribution. A more detailed description of preprocessing in general and the approach used in this work is provided in 2.2. Based on intensity values of T1-wheighted MR images and prior probability maps, a value is assigned to each voxel. This value reflects the probability of containing grey matter (GM), white matter (WM), cerebrospinal fluid (CSF), meninges, non-brain tissue, or background. This process is referred to as tissue segmentation. Of particular interest are the tissues containing neurons, fibers and glial cells, namely GM and WM.

The favored statistical model used in brain morphometry is the general linear model. As the name implies, a combination of independent variables has a linear impact on a dependent variable. In case of brain morphometry, the dependent variables are values derived from MR images by segmentation which reflect a probability to find a given tissue in this voxel. Different models testing for different aspects may be designed, depending on the hypothesis and the composition of the data under study. Creating appropriate contrasts allows for the examination of independent variables' influence on image data, separately or in combinations of several independent variables.

Statistical tests are applied in a voxel-wise manner, resulting in a statistical (parametric) map for each contrast. Of note, a large number of voxels leads to a large number of independent statistical tests, ultimately resulting in a high rate of false positive results. Thus, results need to be corrected for this so-called type-1-error. After having corrected for multiple comparisons, t-maps can be interpreted to reflect the significant relation between the tissue probability likelihood and the parameter of interest.

VBM enables detection of local alterations in brain structure without defining regions of interest beforehand. (Ashburner, 2009, Ashburner and Friston, 2000a, Ashburner and Friston, 2005, Pernet, 2016) Despite recent doubts (Eklund et al., 2016), VBM yields statistically valid results when corrected adequately for multiple comparisons.

1.2.5 Surface-Based Morphometry

While volumetric analyses such as VBM assess regional tissue volume, surface-based approaches allow assessing other features, such as cortical thickness and cortical surface area.

As all these parameters show differing developmental patterns throughout childhood, it was postulated that each parameter at least partly describes different underlying neuronal processes (Wierenga et al., 2014, Vijayakumar et al., 2016). Therefore, alterations in brain development may be missed when analyzing solely one parameter (Wierenga et al., 2014). Signals from subcortical structures determine surface area, whereas cortical thickness rather depends on local factors. Cortical volume (the parameter assessed by VBM) is the product of surface area and thickness. (Rakic, 1995) Other features, such as gyrification and sulcal depth describe the morphology of exposed and unexposed brain surface. Analyses of several cortical parameters allows drawing a more complete picture of morphological alterations of brain anatomy, since surface-based analyses of cortical thickness, gyrification and sulcal depth, as conducted in this paper, complement VBM-analyses. Surface-based parameters are schematically illustrated in Figure 1.

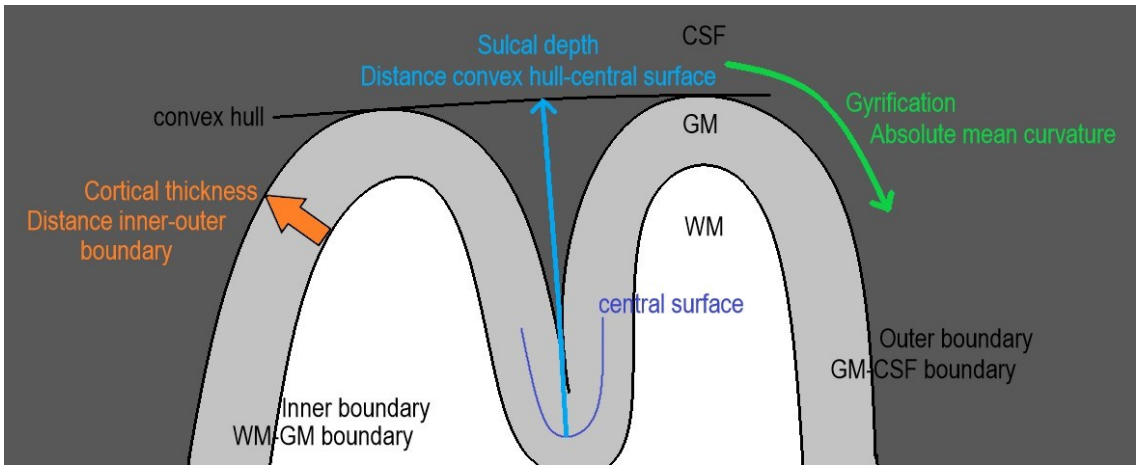

Figure 1: Schematic illustration of surface-based parameters.
Cortical thickness describes distance between inner, white matter (WM)-grey matter (GM) boundary, and outer, GM-cerebrospinal fluid (CSF) boundary. Sulcal depth describes distance between central surface and convex hull. Gyrification describes absolute mean curvature of gyri and sulci. CSF=cerebrospinal fluid, GM=grey matter, WM=white matter. Figure adapted from Dahnke and colleagues (Dahnke et al., 2013).

Cortical thickness describes the thickness of grey matter and, on a cellular level, neuron column's height throughout the cortex (Rakic, 1995). Together with surface area, cortical thickness determines cortical volume (Vijayakumar et al., 2016). Cortical thickness is calculated by determining the inner boundary of GM to WM, and the outer boundary of GM to CSF. In this paper, a computational anatomy toolbox is used, namely CAT12 (developed by Christian Gaser and Robert Dahnke at the departments of Psychiatry and Neurology at Jena University Hospital), a toolbox running on SPM12 (Wellcome Trust Centre for Neuroimaging, University College London. UK). CAT12's approach uses Eikonal equations to calculate each GM voxel's closest distance to WM, i.e. the inner boundary. The maximum value reflects cortical thickness. Since narrow sulci contain only small amounts of CSF, they are at risk of being blurred after smoothing. Voxels can further contain more than one tissue type and may be ascribed to the wrong tissue class (partial volume effect). Consequently, cortical thickness is at risk to be overestimated. Projection-based thickness approaches tackle these issues (Dahnke et al., 2013).
Secondly, gyrification analyses enable a more detailed investigation of early brain development. Gyrification sets in during the fifth month of fetal life and continues

after birth. Since migration of neurons into the cortex occurs between the second and sixth month of pregnancy, it is likely that other factors contribute to gyrification. Primary and secondary sulci develop first, showing a constant pattern throughout all individuals. Tertiary sulci, which still develop postnatally, show a much more diverse pattern. It has been suggested that convolution depends on growth patterns within cortex layers or on compressive stress (Richman et al., 1975).

CAT12's approach calculates the degree of convolution at surface points within 3 mm distance across the whole surface of the brain. From a mesh which models the brain surface, the mean curvature (the change in expected surface trend) is calculated. Sulci result in large negative values whereas gyri result in large positive values. Next, absolute mean curvature is determined resulting in positive values only. Local values are then combined within a region by applying spatial smoothing. (Luders et al., 2006)

Analyses of sulcal depth were also included in this work. Primary sulci, such as the interhemispheric fissure, the Sylvian fissure or the central sulcus, form by 33 weeks of gestation. They are followed by secondary sulci, beginning to form at 31 weeks of gestation, and tertiary sulci. Fetal MRI, MRI of preterm born infants and neuropathological examinations indicated that prominent sulci, like superior temporal, inferior temporal, central, postcentral, precentral and olfactory sulcus, develop at 20 to 32 weeks of gestational age. Therefore, sulcal depth analyses may be sensitive to early impairment in brain development. Especially the development of later-developing sulci, such as temporal sulci at 27 to 32 weeks of gestation, may be influenced by preterm birth. (Dubois et al., 2008b, Garel et al., 2001, Kim et al., 2016a).

Sulcal depth is calculated by applying a convex hull onto the brain surface and calculating the Euclidean distance between the hull and the central surface. CAT12's approach uses squared root transformed sulcal depth to render data more normally distributed. (Gaser and Kurth, 2016)

1.2.6 Alteration of Brain Structure Following Preterm Birth

Throughout the last weeks of pregnancy, rapid brain development is still taking place (Ouyang et al., 2018, Ortinau and Neil, 2015). In preterm born infants, those

crucial stages of development take place outside the safe, providing and nourishing environment of the mother's womb. Thus, it seems very plausible to assume that brain development will be altered in preterm born infants.

Severe brain injury in preterm born infants is already visible in ultrasound examinations through the anterior fontanel after birth. Cranial ultrasound enables detection of IVH, periventricular hemorrhages, post-hemorrhagic ventricular dilatation, periventricular hemorrhagic infarction, classic PVL and abnormalities of cerebral gyrification. MRI is more sensitive to detect small IVH, cerebellar hemorrhages, diffuse periventricular leukomalacia, impaired myelination and abnormalities in posterior fossa. (Benders et al., 2014, Melbourne et al., 2016, van Wezel-Meijler et al., 2010) Brain injury and consecutive morphological abnormalities were shown to be predictive of preterm born infants' long-term neurologic outcome (Anderson et al., 2017).

1.2.7 Global Brain Volume

More sensitive morphometric analysis approaches reveal less overt alterations of brain structure. At term equivalent age, preterm born infants showed reduced total brain volumes of all tissue types, as well as local volumetric alterations of GM and WM in parieto-occipital, sensorimotor, orbitofrontal and premotor regions. CSF was found to increase reciprocally (Thompson et al., 2007). Differences in absolute brain volume of GM, subcortical GM, and WM persisted throughout childhood (Monson et al., 2016). Persisting structural brain alterations in older preterm born children could also be shown, via estimations of brain maturation. The MR images of the subjects were compared to the brain's true chronological age, using the BrainAGE (brain age gap estimation) framework. Those comparisons showed greater discrepancies between estimated and true age of the brain in former preterm born children, than in full-term born individuals (Franke et al., 2012).

1.2.8 Local Brain Volume

In voxel-wise examinations of regional brain structure, several studies found local alterations in GM structure when examining babies (Inder et al., 2005), children (Zubiaurre-Elorza et al., 2011, Soria-Pastor et al., 2009, Kesler et al., 2008),

adolescents (Spencer et al., 2008, Nosarti et al., 2008) and adults (Nosarti et al., 2014, Bauml et al., 2015) with a history of preterm birth. In most studies low birthweight preterm born children and very preterm born children were examined, but some studies also showed reduced grey matter volume (GMV) in low-risk preterm born children (born at 30-34 weeks of gestation) (Soria-Pastor et al., 2009). Recurring regions of reduced GMV were superior, medial and inferior temporal gyrus (Spencer et al., 2008, Nosarti et al., 2008, Nosarti et al., 2014, Isaacs et al., 2004, Kesler et al., 2008, Zubiaurre-Elorza et al., 2011), frontal lobe (Kesler et al., 2008, Nosarti et al., 2014, Nosarti et al., 2008, Zubiaurre-Elorza et al., 2011, Spencer et al., 2008, Bauml et al., 2015), thalamus (Nosarti et al., 2014, Nosarti et al., 2008, Kesler et al., 2008), hippocampus (Isaacs et al., 2000) and cerebellum (Spencer et al., 2008).

1.2.9 Surface-Based Parameters

Similarly, analyses of surface-based parameters showed short-term and long-term alterations of brain structure in preterm born children.

Cortical thickness was both increased and decreased in preterm born compared to full-term born children (Murner-Lavanchy et al., 2014), adolescents (Nagy et al., 2011, Frye et al., 2010, Nam et al., 2015) and young adults (Nam et al., 2015). Studies examining children and adolescents with low birth weight, which were not necessarily born preterm, similarly revealed cortical thickness alterations in children (Solsnes et al., 2015) and adolescents (Martinussen et al., 2005, Bjuland et al., 2013) compared to controls with average birth weight demonstrating that this parameter is not a specific indicator of effects of prematurity, but of early interferences with brain development in more general terms. Regions of cortical thickness increase or decrease differed in between studies. Most frequently, analyses of preterm born or low body weight study participants revealed cortical thickness decreases in temporal (Martinussen et al., 2005, Bjuland et al., 2013, Murner-Lavanchy et al., 2014, Frye et al., 2010, Nagy et al., 2011) and parietal lobe (Martinussen et al., 2005, Bjuland et al., 2013, Solsnes et al., 2015, Nagy et al., 2011), and increase in prefrontal (Martinussen et al., 2005, Bjuland et al., 2013, Solsnes et al., 2015, Murner-Lavanchy et al., 2014, Nam et al., 2015, Nagy et al., 2011) and occipital regions (Martinussen et al., 2005, Bjuland et al., 2013,

Solsnes et al., 2015, Nam et al., 2015). Further, premature preschool children (Phillips et al., 2011), school-aged children (Murner-Lavanchy et al., 2014) and adolescents (Nam et al., 2015) showed delayed cortical thinning. Cortical thinning was described to be a morphologic correlate of physiologic brain maturation in most parts of the brain (Vijayakumar et al., 2016). In the studies mentioned above, it was thus suggested that children with increased cortical thickness exhibited delayed brain maturation.

Moreover, whole-brain gyrification index, as well as local sulcus depth, was described to be decreased at term equivalent age in the insula, superior temporal sulcus and pre- and postcentral areas (Engelhardt et al., 2015). In accordance with those results, increasing abnormality of the newborn brain was associated with decreasing gyrification index (Moeskops et al., 2015). Results of studies during childhood, however, contradict each other. On the one hand, at the age of eight years, local increase of gyrification in temporal lobe was found in preterm born children (Kesler et al., 2006). On the other hand, smaller whole-brain gyrification index and shallower sulcal depth was found in very preterm born children at 7 years of age (Zhang et al., 2015). Alterations of gyrification seem a likely consequence of prematurity, considering that cortical folding takes place at the end of second and during third trimester of pregnancy (Richman et al., 1975). Gyrification was described to peak during infancy; hence alterations in gyrification can be seen as a marker of early disorders in brain development (Raznahan et al., 2011, Kim et al., 2016b).

Formation of sulci in fetal brains was described to begin after 2 months of pregnancy and to continue after birth. Intrauterine MRI enabled detailed dating of sulcal formation. Formation of sulci rapidly increased at 30 weeks of gestation (Dubois et al., 2008b). MRI of preterm born infants showed that sulci deepened at 26 to 42 weeks postmenstrual age by 0.04 mm per week on average (Kim et al., 2016a). When comparing cortical folding of extremely preterm born infants, to cortical folding of preterm born infants born at 32 to 34 weeks of gestation, impaired cortical folding in extremely preterm born children was observed in right middle temporal lobe, superior frontal lobe and in sulci surrounding occipitoparietal fissure. Although active formation of sulci is still ongoing during

the phase when preterm children are born, few studies have focused on the investigation of sulcal depth in preterm born individuals throughout childhood. One study of 7-year-old preterm born children reported decreased sulcal depth in superior temporal gyri compared to full-term born children (Zhang et al., 2015). Gimenez and colleagues focused on orbitofrontal sulci and reported decreased sulcal depth of secondary sulci in preterm born adolescents (Gimenez et al., 2006). Zubiaurre-Elorza and colleagues found decreased surface measure of superior temporal sulcus when investigating maximum depth of four selected sulci (Zubiaurre-Elorza et al., 2009). Due to the analysis approach being relatively new, fewer studies are available and no clear pattern and/or interpretation has yet emerged as to the value of this parameter in preterm-born subjects.

1.2.10 Impact of White Matter Damage on Cortical Structure

Regarding the etiology of altered brain structure in preterm born individuals, most authors agree that PVL is the main initiating factor. Pre-oligodendrocytes, the myelinating cell population in preterm born infants, are damaged, leading to impaired myelination, axon degeneration and, subsequently, to attenuated neural signals into the cortex. Further, axons may also be injured primarily, followed altered transmission of neural signals, as described above. Thirdly, neural injury to the thalamus, the main gateway into the cortex, likewise could diminish cortical input. Fourthly, primary injury to subplate neurons, which play a role in the establishment of long-distance connections, would similarly attenuate neural signal into the cortex. Attenuated signals from subcortical areas to the cortex subsequently could lead to impaired cortical development and possibly cortical atrophy. (Volpe, 2009)

1.2.11 Altered Brain Structure in Preterm Born Children Correlates with Cognitive Impairment

Altered brain structure in preterm born children was found to correlate with cognitive impairment. Moreover, reduced GMV was consistently shown to be a marker of cognitive impairment of preterm born infants at term equivalent age (Monson et al., 2016), in one-year-olds (Inder et al., 2005) and in 7-year-olds (Monson et al., 2016). In 9-year-old preterm born children, reduced GMV in

temporal and parietal lobe correlated with IQ (Soria-Pastor et al., 2009). Regarding surface-based parameters, functional assessment at term equivalent age correlated with cortical surface area at birth of preterm born infants (Dubois et al., 2008a).

1.3 Human Cytomegalovirus

1.3.1 Epidemiology

Human cytomegalovirus (hCMV) belongs to the family of herpesviridae and is also known as human herpesvirus 5 (HHV5). It is a DNA virus with polymorphic genome, varying between hCMV strains. Following primary infection, herpesviridae are known to persist in numerous tissue types within the human organism, most prominently in CD 34 positive cells of the bone marrow, salivary and mammary glands, kidneys and liver. This latent infection is usually controlled by the immune system but can be reactivated in case of immunosuppression or times of great stress. HCMV is transmitted horizontally, in close personal contact, via body fluids (like saliva, urine and breast milk), via blood transfusion and transplanted organs. Further, intrauterine vertical transmission is possible. Following infection, hCMV is secreted in body fluids. Incidentally, secretion is especially high in young children, thus, people in contact with young children are at high risk of infection with hCMV. (Suerbaum et al., 2016, Goodrum et al., 2002) HCMV seroprevalence in adults is >90% in developing countries like Nigeria (Neirukh et al., 2013, Hamid et al., 2014), >70% in Southern Europe (Lopo et al., 2011) and 40-50% in Western Europe. In Western European countries, seroprevalence is higher in females, in people with low socioeconomic status and among people with non-Western European background. (Enders et al., 2012, Korndewal et al., 2015, Pembrey et al., 2013)

1.3.2 Infection in Children and Adults

Only a small percentage of hCMV infections in immunocompetent children and adults are clinically apparent. Clinical symptoms may include fever, mild hepatitis, splenomegaly, fatigue, lymphadenopathy and atypical lymphocytosis. Severe cases may show interstitial pneumonia, anemia and chorioretinitis. In case of

immunodeficiency, either iatrogenic immunosuppression or in HIV infection, primary infection with hCMV, as well as reactivation of hCMV, may cause severe clinical symptoms, including organ dysfunction. First-line treatment of severe cases is ganciclovir or valganciclovir. (Suerbaum et al., 2016)

1.3.3 Congenital Infection

Congenital hCMV infection is the most common non-genetic cause of innate neurodevelopmental disability in children (Boppana et al., 2013) Thus, congenital hCMV infection has considerable social impact. Prevalence of congenital hCMV infection was estimated to be 0.5-1% of livebirths in industrialized nations (de Vries et al., 2011, Naing et al., 2016) and ranged up to 6% in developing countries (Lanzieri et al., 2014). In these cases, intrauterine infection occurs via vertical transmission of hCMV. In the first and second trimester, approximately one third of primary maternal hCMV infections lead to congenital hCMV infection. Transmission rates increased over 70% in the third trimester (Enders et al., 2011). Until recently, predominantly *primary* infection of hCMV in pregnant women was said to cause congenital hCMV infection, whereas recurrent infections were considered to be mostly harmless (Kenneson and Cannon, 2007). Recent research, however, suggested that congenital hCMV infection following *non-primary* infection of seropositive mothers may be as prevalent as primary infection (Townsend et al., 2013, Wang et al., 2011, Britt, 2015). Nevertheless, publications state that maternal immunity to hCMV still has a protective effect against hCMV transmission but not against clinical manifestation in case of a fetal infection (Permar et al., 2018, Plotkin and Boppana, 2019, Simonazzi et al., 2018).

Unborn infants were particularly vulnerable to long-term sequelae when infections occurred during first and second trimester (Foulon et al., 2008, Enders et al., 2011, Oosterom et al., 2015). Outcome of congenital hCMV infection varies widely, from unaffected, normal development to intrauterine death. About 10% of infants born with congenital hCMV infection were described to be symptomatic. The most common symptoms were hepatosplenomegaly, jaundice, petechiae and neurologic abnormalities. Hepatobiliary symptoms were likely to disappear over time, whereas neurologic sequelae in many cases were permanent. The

most-frequent long-term sequela was sensorineural hearing loss, affecting two thirds of children which had been symptomatic at birth. Initially asymptomatic children developed sensorineural hearing loss in 10% on average and hearing further deteriorated in half of children affected. (Naing et al., 2016, Buxmann et al., 2017, Goderis et al., 2016, Kenneson and Cannon, 2007, Townsend et al., 2013, Boppana et al., 2013)

In more than half of the children symptomatic at birth, congenital hCMV infection resulted in radiologic brain abnormalities, depending on time of infection. Following infection during the early gestational weeks, severe brain malformations such as schizencephaly and lissencephaly occurred. Later, mid-gestation infections resulted in local cortical overgrowth in the form of polymicrogyria. Late third trimester infections did not lead to overtly appreciable brain malformations. (Barkovich and Lindan, 1994) Apart from that, children with congenital hCMV infection showed a wide range of further abnormalities, classically including WM calcifications, infarctions, cleft dysplasia, heterotopia, cerebellar hypoplasia and ventriculomegaly. (Jansen and Andermann, 2005, White et al., 2014, Naing et al., 2016)

On a cellular level, four main pathomechanisms of congenital hCMV infection were discussed. Brain development may be disrupted by, firstly, loss of neuronal stem cells, which are predominantly affected by hCMV and consequently succumb to intracellular viral replication. Secondly, stem cell migration into the cortex and stem cell differentiation may be disrupted by hCMV infection. Thirdly, infection of and damage to astrocytes by hCMV may be followed by impaired neural fiber isolation. Apart from that, astrocytes and other glial cells play an important role in guidance of neural differentiation and synaptogenesis. Hence, damage of astrocytes may impair the establishment of neural networks. Fourthly, immune response may play a role, as congenital hCMV infection leads to widespread activation and migration of $CD8^+$ cytotoxic T-cells into the brain. Released cytokines are neurotoxic and may influence neural and glial differentiation and migration. (Gabrielli et al., 2012, Teissier et al., 2014, Cheeran et al., 2009)

In summary, 40-50% of symptomatic children and 10-25% of initially asymptomatic children later showed neurocognitive impairment (Dreher et al., 2014, Townsend et al., 2013) which underlines the severe detrimental effect of congenital hCMV infections on the developing brain.

1.3.4 Postnatal Infection in Full-Term Born Children

Reactivation of hCMV may occur in times of great stress (Rector et al., 2014) and is consequently observed in many mothers following childbirth in general (Dworsky et al., 1983, Wang et al., 2015) and preterm labor in particular (Hamprecht et al., 2001, Kurath et al., 2010).

Most studies on transmission of hCMV via breast milk focused on preterm born infants. In studies with mixed groups, including both full-term born and preterm born infants, around 30% of seropositive mothers shed hCMV in their breast milk. One third to two thirds of the exposed infants were infected with hCMV postnatally. However, it is not discernible whether those infants infected were born preterm or full-term (Dworsky et al., 1983, Wang et al., 2015). Population studies showed that hCMV seroprevalence among young children correlated with the rate of breastfeeding mothers in those populations, which may indicate the relevance of breast milk transmission also in full-term born children (Stagno and Cloud, 1994).

The majority of full-term born infants neither showed clinical symptoms while shedding hCMV, nor did they develop any long-term neurologic sequelae (Gentile et al., 1989, Kumar et al., 1984, Paryani et al., 1985, Johnson et al., 1986, Stagno and Cloud, 1994). Only few cases of term born, normal body weight children with symptomatic postnatal hCMV infection have been reported. Those postnatally infected full-term born infants showed hepatitis (Vancikova et al., 2004, Hasosah et al., 2012), pneumonitis (Stagno et al., 1981), gastrointestinal pesudoobstruction (Hendriks et al., 2013) or a chorioretinal scar (Kanik-Yuksek et al., 2014), respectively. Even though rare cases may show clinical manifestations, in most cases postnatal hCMV infection in term born infants is now accepted to be harmless in the short and in the long term. It is assumed that intrauterine transmission of maternal anti-hCMV antibodies, may protect term

born children from postnatal infection with hCMV, or at least from the negative consequences (Bryant et al., 2002, Saso and Kampmann, 2020).

1.3.5 Early Postnatal Infection in Preterm Born Infants

It is common knowledge that preterm born infants in their first months of life can be crucially endangered by infections of any kind. One of those is early postnatal infection with hCMV; “early” meaning occurring in the first months of life until three months corrected age (Maschmann et al., 2001).

A range of studies exists on the transmission of hCMV to preterm born infants via breast milk. The rate of hCMV secretion in breast milk of hCMV-seropositive mothers to preterm born infants ranged from 66 to 96% (Kurath et al., 2010). 6-59% of the preterm born infants exposed to hCMV-positive breast milk were infected by hCMV postnatally (Kurath et al., 2010), depending on the viral load and the duration of viral shedding in breast milk (Jim et al., 2009, van der Strate et al., 2001). Transmission rates of the original cohorts of this work’s study group have also been published. In 96%, mothers of preterm born infants shed hCMV DNA in their breast milk and 37% of exposed preterm born infants were postnatally infected with hCMV (Hamprecht et al., 2001). Since most maternal antibodies are transmitted in the third trimester (Simister, 2003), most preterm born infants, especially very preterm born infants, are not as effectively protected against postnatal hCMV infection as term born infants are. The majority of preterm born infants infected with hCMV did not develop any clinical symptoms (Neuberger et al., 2006). Rates of symptomatic hCMV infection varied greatly between studies. Most studies reported rates of approximately 15%, ranging from 0% up to more than 30%. (Yeager et al., 1983, Miron et al., 2005, Lanzieri et al., 2013, Kurath et al., 2010, Josephson et al., 2014, Bryant et al., 2002) However, several cases of severe postnatal hCMV infections in this population have been described. Most frequently, severe cases showed pneumonitis and/or respiratory distress, hepatosplenomegaly, elevated liver enzymes, hyperbilirubinemia, thrombocytopenia, colitis and sepsis-like symptoms. Risk of BPD was significantly higher in preterm with an early-postnatal hCMV infection, than those without such an infection (Kelly et al., 2015). In some preterm born infants, hCMV infection was fatal. (Stagno et al., 1981, Hamele et al., 2010, Takahashi et al.,

2007, Fischer et al., 2012, Anne-Aurelie et al., 2016, Yeager et al., 1983, Hamprecht and Goelz, 2017)

Autopsy of one fatal case showed “owl eyes”, typical for an infection with hCMV, in lung, liver, kidneys, pancreas and adrenal glands. The brain was affected as well, showing signs of hCMV encephalitis and meningeal hemorrhages. (Anne-Aurelie et al., 2016) In summary, hCMV in some preterm born infants obviously can cause serious systemic morbidity, whereas in other cases, preterm born infants seem to be able to cope with an acute hCMV infection without developing any symptoms.

1.3.6 Long-Term Consequences of Early Postnatal Infection in Preterm Born Children

Follow-up data of preterm born children postnatally infected with hCMV is sparse. Case reports of preterm born children with severe postnatal hCMV infection reported transient hearing impairment and global cognitive delay in one of two cases (Fischer et al., 2012, Takahashi et al., 2007). Outcome of preterm born children at 12 and 24 months did not show any significant impairment in preterm born children with postnatal hCMV infection (Jim et al., 2015). Further, follow-up at two to four and a half years of preterm born children with mostly asymptomatic hCMV infection showed no significant differences in hearing, cognitive and neuromotor skills (Vollmer et al., 2004). Testing at mean age of six and a half years showed that preterm born children with early postnatal hCMV infection did score lower in all tests, however, differences were not significant. (Goelz et al., 2013) Comparably, in a Dutch study, preterm with early postnatal hCMV infection scored lower in cognitive testing, but no *significant* difference in cognitive and motoric development up to six years of age was found (Gunkel et al., 2018). At median age of eight years, preterm born children with early postnatal hCMV infection scored significantly lower in simultaneous processing of cognitive testing and in motor function testing, than preterm born children without early postnatal hCMV infection (Bevot et al., 2012). Further follow-up at mean age of roughly 14 years of age showed that preterm born children with early postnatal hCMV infection scored significantly lower in IQ testing than preterm born children without hCMV infection (Brecht et al., 2015). Follow-up at school-age thus provided good

evidence for long-term cognitive sequelae of early postnatal hCMV infection in preterm born children, even though the initial infections were mostly asymptomatic.

Cranial ultrasound of very preterm born children with mostly asymptomatic postnatal hCMV infection revealed cerebral abnormalities in more than 50% of very preterm born children. Ultrasound revealed lenticulostriate vasculopathy and germinolytic cysts. The clinical relevance of those findings is still controversial, however. (Nijman et al., 2012a, Nijman et al., 2012b)

The first publication on MR imaging of very preterm born children with early postnatal hCMV infection, at term-equivalent age, described reduced fractional anisotropy in WM of occipital lobe, indicating early microscopic WM alterations. (Nijman et al., 2013)

FMRI data of preterm born children with and without hCMV infection, of the same cohort as investigated in this work, has previously been published. During language tasks, group differences in left hippocampus were mainly caused by those preterm born children who had suffered from an early postnatal infection with hCMV. Further, right anterior cingulate gyrus showed activation increase in preterm born children with hCMV infection compared to preterm born children without early postnatal hCMV infection. Increased activation was attributed to higher cognitive demand and greater efforts during language tasks in preterm born children with a history of early postnatal hCMV infection. Those results thus indicated that hCMV infection after birth in preterm born children caused functional impairment in the brain, which was still traceable in late childhood. (Dorn et al., 2014)

1.3.7 Prevention of Early Postnatal Infection in Preterm Born Infants

Even since hCMV viruses have been isolated from breast milk in the 1970s (Hayes et al., 1972), it is common knowledge that breast milk is one of the leading postnatal transmitters of hCMV. Reactivation of hCMV in seropositive mothers seems to play a great role, and reactivation and viral shedding may even be restricted to breast milk (Hamprecht et al., 2008). To protect newborns and especially preterm born infants from hCMV infection, methods to inactivate hCMV viruses have been applied. Freezing did preserve enzymatic activities and

nutritive factors but does not fully eradicate the virus. Consequently, transmission of hCMV was not successfully prevented, as cases of hCMV infection of preterm born infants after freezing of breast milk indicate. Holder pasteurization for 30 minutes in a water basin of 62.5°C effectively inactivated hCMV viruses, but also inactivated enzymes as a negative side effect. Short-term inactivation for five seconds at 62°C yielded the most promising results: Viral effectivity ceased, transmission of hCMV was significantly reduced, and enzyme activity as well as hCMV antibody function was at least partly preserved. (Hamprecht et al., 2004, Hamprecht and Goelz, 2017, Bapistella et al., 2019, Maschmann et al., 2019)

The public health institute in Germany (Robert Koch-Institut) advised against breastfeeding by seropositive mothers of preterm born infants in NICUs due to a higher risk for infection-related complications or sequelae. (Robert Koch-Institut, Milde-Busch, 2014) Only very recently, pasteurization of breast milk from hCMV-seropositive mothers given to the more immature preterm born infants was officially recommended for Austria (Haiden et al., 2019). However, other countries are still discussing the necessity of preventive measures of early postnatal hCMV infection in preterm born babies (Wright and Permar, 2015, Anne-Aurelie et al., 2016). Further, also in Germany, these measures are not followed unanimously as recommended by more than one fourth of NICUs (Buxmann et al., 2010). Thus, further research on the consequences of early postnatal hCMV infection in preterm born infants could influence this ongoing discussion on the necessity of preventive measurements.

1.4 Research Questions

There is a large body of literature describing that preterm born children show short- and long-term global and local structural brain alterations. Further, structural alterations were described to correlate with poorer cognitive abilities of preterm born compared to full-term born children. On the other hand, neuropsychological testing of preterm born children with early postnatal hCMV infection compared to preterm born children without such an infection showed additional neurocognitive impairment in preterm born children having suffered from an early postnatal hCMV infection. However, no study to date ever analyzed the long-term influence of early postnatal infection with hCMV on GM structure of

preterm born children. This work analyzed whether early postnatal infection with hCMV had a long-term influence on brain structure of former preterm born children. We focused on global brain volumes and regional GMV, as well as surface-based parameters, since these two analytic approaches must be expected to assess different aspects of potentially altered brain development. As the transmission of hCMV via breastmilk (and, thus, early postnatal infection with hCMV itself) could be prevented, further studies regarding the potential influence of early postnatal hCMV infection on preterm born children seem most important. Considering that an early postnatal infection with hCMV has been shown to influence the cognitive abilities of former preterm born children, we hypothesized that early postnatal infection with hCMV would have a long-term influence on brain structure of former preterm born children. Specifically, we expected GMV to be decreased in former preterm born children with the effect being more pronounced in preterm born children with early postnatal hCMV infection. We further expected the surface-based parameters to be more abnormal in preterm born children with early postnatal hCMV infection than in preterm born children without such an infection, due to the viruses known potential to interfere with cortical development in congenital hCMV infection.

2 Subjects and Methods

2.1 Study Design

Participants of the present work were part of a long-term follow up study investigating the consequences of an early postnatal infection with hCMV via breast milk in preterm born participants. Some of the participants included in this work were also part of previous studies (Bevot et al., 2012, Brecht et al., 2015, Dorn et al., 2014, Goelz et al., 2013). Participating preterm born children (PT) were born between July 1995 and September 1999 and were treated in the NICU at the Children's Hospital of the University of Tübingen. Participants were born ≤ 32 weeks of gestation and/or weighed ≤ 1500g at birth. The study only included participants that had not acquired an infection with hCMV congenitally, which was ruled out by examining ear and throat swabs and urine in the first week following their birth. (Hamprecht et al., 2001). Furthermore, their mothers' serostatus was obtained. To detect early postnatal hCMV infection, participants' urine as well as their mother's breast milk was examined biweekly during hospital stay via PCR analyses. If hCMV strains were detected in the preterm infant's urine during hospital stay and serologic testing was positive, these participants were defined as having acquired an early, pre-discharge postnatal infection with hCMV. Thus, they were assigned to group "preterm hCMV+" (PT_{hCMV+}). Preterm born infants were defined as hCMV negative (PT_{hCMV-}), either if the mother was seronegative or, if the mother was seropositive, infants were tested hCMV-negative via urine and serologic testing up to the age of three months corrected age when the last testing was done. Since urine was systematically testes biweekly during hospital stay and at three months corrected age, we feel reasonably certain that we did not miss any hCMV infection and that all preterm born infants of the negative group were indeed hCMV negative. In addition, serostatus of all mothers and babies was obtained. If hCMV strains were detected in the participants' urine within two to twelve weeks of life or blood samples were hCMV-seropositive, in addition to a positive urine culture, those participants were defined as having acquired an early postnatal infection with hCMV. Thus, they were assigned to group "preterm hCMV+" (PT_{hCMV+}). Participants who did not suffer from an early postnatal infection with hCMV, meaning negative PCR and culture of urine

samples up to three months corrected age, are consequently considered “preterm hCMV-” (PT $_{hCMV-}$). For further detailed information on the microbiological tests see (Hamprecht et al., 2001), (Neuberger et al., 2006) and (Maschmann et al., 2001). All former preterm born participants were identified from hospital records and approached in writing. In this way, 94 PT were included; 50 hCMV- and 44 hCMV+. Data concerning the neonatal history was also collected.

Members of the full-term born control group (FT) were recruited in 2012 by public announcements, advertisement in the press and by word of mouth. They were required to show no signs or history of a postnatal infection with hMCV, meaning on the one hand, no neurological or psychiatric disorders, hearing deficits or cognitive impairment. On the other hand, meaning that members of the control group were required to have no history of neonatal infection, hepatosplenomegaly, thrombocytopenia or prolonged jaundice possibly indicating a neonatal hCMV infection. Unfortunately, there was no data on postnatal hCMV-serostatus or urine status of FT available. Further, all participants were required to have no MR contraindications. (Brecht et al., 2015, Dorn et al., 2014, Wilke et al., 2014)

The study was approved by the Ethics Committee of the University of Tübingen’s Faculty of Medicine (file number 215/2009B01). Written informed consent was obtained from at least one parent and all children gave verbal assent.

2.1.1 Demographic Details of the Study Population

Demographic data of participants’ gender and age at assessment were collected before neurological assessment and image acquisition. Additionally, information on relevant neonatal information for the PT group (gestational age, birth weight, singlet or multiplet, incidence of ICH, NEC, BPR, ROP) were collected from the original hospital charts. Overall, a total of 75 subjects could be included, 37 in group PT, of which 14 were PT $_{hCMV+}$ and 23 were PT $_{hCMV-}$, and 38 in group FT. More details are provided in Table 1, Table 2 and Table 3 in the results section.

Maternal education (ME) was described to be a relevant factor for outcome of preterm born children (Patra et al., 2016) and scaled to reflect years of education, according to the mother’s highest degree (9: not specified/graduated from “Hauptschule”; 10: graduated from “Realschule”; 13: graduated from

“Gymnasium”; 3 added for vocational training; 5 added for university degree) Consequently, final numbers range from 9 to 18 (18: graduated from “Gymnasium” plus obtaining a university degree).

2.1.2 Neurological and Neuropsychological Assessment

All participants were assessed by child psychologists of the Children’s University Hospital Tübingen. The HAWIK IV (the German version of the Wechsler Intelligence Scale for Children) was used to assess participants’ cognitive abilities. A standardized full-scale IQ was obtained, as well as scores in several subcategories (verbal comprehension, perceptual reasoning, working memory and processing speed) (Petermann and Petermann, 2008). Moreover, handedness was determined by the Edinburgh Handedness Inventory (EHI) (Oldfield, 1971).

2.2 MR Image Acquisition and Preprocessing

2.2.1 Image Acquisition

All participants were scanned at the University Hospital Tübingen using a 1.5 Tesla MR-scanner (Siemens Avanto, Erlangen, Germany) with a 12-channel head coil. Structural T1-wheighted 3D images were acquired consisting of 176 sagittal slices of 1 mm thickness with a matrix size of 256 × 256 and no gap, yielding voxel size of 1 × 1 × 1 mm^3. Echo time was 2.92 s, repetition time was 1300 ms. (Wilke et al., 2014, Dorn et al., 2014).

2.2.2 Software Details

Data was preprocessed using SPM12 (Wellcome Trust Centre for Neuroimaging, University College London, UK) running on Matlab (R2014b, The Mathworks, Natick) and CAT12, a computational anatomy toolbox (Christian Gaser and Robert Dahnke, Departments of Psychiatry and Neurology at Jena University Hospital) running on SPM12 (Gaser, 2018).

2.2.3 Coregistration and Reorientation

Although participants’ heads are in a fixed position in a mask during image acquisition, the heads’ and therefore the brains’ positions differ. In order to

achieve better starting estimates for the following automated processing steps, all images need to be reoriented.

Firstly, images were coregistrated automatically by SPM12 using a SPM T1 template. Those were further reoriented manually. As a reference point, the image volume "origin" was set to the anterior commissure.

2.2.4 Creating a TOM Template

When MR images were acquired, our participants were on average 13.6 years of age. By creating a TOM (template-o-matic) template, confounding effects of an adult template can be avoided. TOM is a toolbox running in SPM8 (Wellcome Trust Centre for Neuroimaging, University College London, UK) to create pediatric templates, matching the studied group in age and gender. Originally, the reference data consists of 404 healthy children aged 5 to 18 years. In the "matched pairs approach", a reference map for each subject is created depending on the subject's gender and age in months, which is then combined with all other maps to generate one final template (Wilke et al., 2008). For the first segmentation cycle, a TOM template, matching our initially 83 subjects, was created.

2.2.5 Affine Normalization and Segmentation

Images were affine registered and segmented into GM, WM and CSF. Those preprocessing steps were performed using CAT12 which uses a revised version of SPM12's. One run combines several steps of preprocessing. In the process, the images were initially skull-stripped. Then they were globally registered to a standard space, correcting the brain's overall size and position. This registration is called an affine or linear normalization. For this process the TOM template, matching our initially 83 datasets, was used.

During segmentation, each voxel is assigned to one tissue class (GM, WM, CSF, meninges, non-brain tissue and surrounding material). Throughout those steps, CAT12 does not use any priors. By analyzing the intensity information of each voxel, voxels are assigned to one type of tissue class. Since GM intensities vary in different brain regions (e.g. between motor cortex and thalamus), the amount of GM is often underestimated. To minimize these effects, CAT12 includes a

“local adaptive segmentation” by which local intensities are transformed. Secondly, data is segmented via a hidden Markov random field (HMRF) approach, which assumes that neighboring voxels are expected to be of the same tissue class. The “Markov random field” (MRF) energy of the 26 surrounding voxels is calculated, resulting in a probability of a voxel belonging to a specific tissue class. In this way, isolated voxels surrounded by voxels of another tissue class, which are therefore most likely noise artefacts, can be corrected. Especially if noise level is above 3-5%, results will be more accurate when HMRF is applied (Wilke and Holland, 2008a, Gaser and Group, Gaser and Kurth, 2016). The modified version of segmentation with the help of HMRF is using an adaptive maximum a posterior (AMAP) segmentation.

Furthermore, assuming that not all voxels contain solely one tissue class but may consist of two types, CAT12 involves a “partial volume segmentation” which adds two mixed classes, GM–WM and GM–CSF. In this way, segmentation results benefit especially at the GM/WM and the GM/CSF interface.

Moreover, during iterative preprocessing, images are warped locally to the corresponding tissue prior. Thus, local brain regions may either increase or decrease in size and, thus, volume. Those non-linear effects are re-integrated into the final maps by modulation using a “Jacobian determinant” of the spatial deformation field. In this way the regions may still be compared regarding their volume.

All aspects of the segmentation algorithm are combined into a Bayesian framework, a statistical framework that allows to maximize the a posteriori probability. (Gaser and Kurth, 2016)

2.2.6 Quality Check

In order to acquire accurate MR images, it is essential that subjects lie still during scanning. Especially for children, this is a demanding task (Wilke and Holland, 2008a). Assuming that not all participants were able to hold still while scanning, images need to be checked systematically regarding noise and movement artefacts.

As a first step, images were inspected visually, and all images of clearly insufficient quality were removed. As a second step, data was checked with the

help of CAT12 creating a sample correlation matrix. In this way, data which deviated substantially from the whole sample was apparent. Of 83 datasets, eight needed to be excluded during these data quality steps. Consequently, 75 datasets remained for ultimate analyses.

Data on image quality was extracted from the report files of final segmentation. A comprehensive image quality rating (IQR) was chosen which combines a contrast-to-noise ratio, an inhomogeneity-to-contrast ratio and the root mean square of the image resolution IQR ranges from 0.5 to 10.5; the lower the IQR, the better the image quality. (Gaser and Kurth, 2016, Gaser, 2018)

2.2.7 Creating a DARTEL Template

Results of segmentation depend on the chosen template. To avoid any confounding effect of unsuitable reference data, to achieve a higher overlap between corresponding structures and to base processing solely on the group under study, a custom DARTEL template was created. DARTEL is a fast diffeomorphic image registration algorithm based on Lie algebra, assuming that an average of the investigated sample would be a better template than any other (Ashburner, 2007). The DARTEL template is an average of the studies' samples and is created in an iterative process. In every cycle, an average of all images is created to which the images are then warped in the following iteration. From those warped images, the next average is generated, to which the images are once again warped. In this way, the average template becomes crisper after each cycle. The template used in our final segmentation passed the recommended number of six cycles (Ashburner, 2007). The use of DARTEL templates was evaluated in previous comparative studies and was declared one of the highest-performing spatial normalization methods (Klein et al., 2009, Yassa and Stark, 2009).

2.2.8 Final Processing

For the final normalization and segmentation of the 75 remaining datasets, the CAT12 processing steps described above were repeated using a newly-generated TOM template in the first step and the study-specific DARTEL template in the final round of spatial normalization and segmentation. This

procedure ensures that the results were obtained with a minimum amount of bias from an inappropriate reference population.

2.2.9 Extracting Global Volumes

The term “total intracranial volume” (TIV) describes the volume within the cranium, namely the sum of GM, WM, and CSF. Data of TIV as well as GMV, white matter volume (WMV) and volume of cerebrospinal fluid (CSFV) were extracted from automatically generated CAT12 report files after segmentation. While manual measurement of TIV is still considered the gold standard, SPM12’s approach was shown to be the most effective substitute for manual measurement (Malone et al., 2015).

2.2.10 Smoothing

Finally, images are smoothed to facilitate comparison between subjects’ images, to eliminate artefacts, and to improve results of statistical tests by rendering the image data more normally distributed. With the help of an isotropic Gaussian smoothing kernel, intensities of the surrounding voxels are considered and included into the voxel’s intensity value (Ashburner and Friston, 2000b). In our study, a Gaussian kernel with a “full width at half maximum” (FWHM) of 6 mm was applied.

2.2.11 Extracting Surface Parameters

Data on cortical thickness, local gyrification and sulcal depth was again obtained by using CAT12. These surface parameters require a larger smoothing kernel. They were smoothed with a Gaussian filter of FWHM = 20 mm, according to recommendations which are based on the average distance between sulci and gyri of 20-30 mm, exploiting the matched filter theorem (Luders et al., 2006).

2.3 Statistics

Data was divided into four groups corresponding to the participant’s main characteristics: FT, all PT, PT $_{hCMV-}$ and PT $_{hCMV+}$, respectively.

2.3.1 Demographic and Neuropsychological Data

Statistical analysis of demographic data was conducted in SPSS 23 (IBM Corporation, Armonk, New York, USA). Due to small sample sizes, Chi-squared tests were conducted to test for group differences of dichotomous, independent variables, i.e. gender, handedness and the occurrence of twins, ICH, NEC, BPD, and RP.

Group differences in image quality, age at assessment, gestational age, birth weight, IQ and maternal education were investigated by applying Mann-Whitney U tests.

Significance was assumed at $p<0.05$, and results were Bonferroni-corrected for multiple comparisons.

2.3.2 Global Volumes

Statistical Analysis of global volumes was conducted in SPSS 23. Due to small sample sizes, Mann-Whitney U tests were used to compare groups FT / PT and PT_{hCMV-} / PT_{hCMV+}. Again, significance was assumed at $p<0.05$ and results were Bonferroni-corrected for multiple comparisons

2.3.3 Data of Voxel-Based Morphometry

In VBM analyses, the four defined groups were considered to be independent from each other, and variance was assumed to be unequal in order to be statistically most robust. The framework of the general linear model (GLM)(Scott et al., 2014) was used to investigate local GM differences between groups. Global differences were corrected by global scaling (see following, 2.3.4). Age, age squared (0) and gender (2.3.5) were included as covariates of no interest. Results were corrected for multiple comparisons by threshold-free cluster enhancement (TFCE) and family-wise error rate (FWE) (see 2.3.7). All results of VBM are shown in neurological convention, i.e., left in the image is left in the brain.

2.3.4 Correction for Global Differences by Global Scaling

When aiming to analyze local differences in brain structure, correction for global differences is inevitable, since global brain volume was described to be influenced by various genetic and environmental factors (Malone et al., 2015,

Barnes et al., 2010, Joel et al., 2015, Peters et al., 1998). As advised by the CAT12 Manual (Gaser and Kurth, 2016) and Christian Gaser (personal communication), global scaling was chosen to correct for TIV. Global scaling divides each voxel by the global mean. In this way, only local effects, which exceed global differences, are analyzed. However, the model assumes that global differences affect all voxels of the brain in the same way. (Peelle et al., 2012, Pernet, 2016) Global scaling can be included in the model batch in CAT12 by entering TIV values as "global calculation" values. Images were then proportionally normalized and scaled to a value of 50. After scaling, the absolute unscaled threshold for including a voxel (default 0.1) was adjusted as follows: new threshold = $\frac{0.1 \times 50}{TIV}$.

2.3.5 Correction for Gender Differences

Gender was entered as a binary covariate in the GLM. Male and female brains are known to differ in absolute size (Peters et al., 1998), in the amount of cortical neurons (Pakkenberg and Gundersen, 1997) and in local GMV in VBM (Joel et al., 2015). Further, brain structure was described to be influenced by both androgens and estrogens (Woolley et al., 1996, Yankova et al., 2001, Martini and Melcangi, 1991). Throughout adolescence, girls' brain development showed faster increases and earlier maxima than boys' brains (Groeschel et al., 2010). In PT, not only short-, but also long-term outcome is described to depend on the child's gender (Kesler et al., 2008). However, it was questioned whether additional correction of gender differences after TIV correction in voxel-based morphometry was necessary (Henley et al., 2010, Pell et al., 2008, Brain Development Cooperative Group, 2012). Further, it was unclear whether gender had an impact on cortical thickness (Wierenga et al., 2014, Frye et al., 2010, Vijayakumar et al., 2016). Gyrification, in contrast, was shown to differ between genders in children (Raznahan et al., 2011) and adults (Luders et al., 2006). Morphology of brain sulci as well has been described to differ between male and female adults (Leroy et al., 2015, Sun et al., 2015), but it is discussed that sulcal differences merely resulted from differences in brain size (Im et al., 2008).

In order to ensure comparable results in all analyses and to rule out a possible bias due to gender differences, gender was included as a covariate in all GLM analyses.

2.3.6 Correction for Differences in Age

As a second covariate, age was included in the GLM. Correction of age was necessary because groups differed in median age, as mentioned in 3.1.1 (FT: 12.1 years, PT $_{hCMV-}$: 14.9 years, PT $_{hCMV+}$: 14.8 years). Previous studies showed that changes in brain structure were non-linear: changes in GMV rather resembled an inverted U-shaped curve. In the early years of life, GMV increases at first rapidly, followed by a slow increase and a peak during early to mid-adolescence, varying in the different lobes of the brain. Afterwards, GMV declined slowly. At approximately 1 year of life, the child's GMV had already reached 80% of the maximum GMV (Groeschel et al., 2010, Giedd et al., 1999). Development of cortical thickness has been described as decreasing linearly (Wierenga et al., 2014) as well as decreasing and increasing non-linearly (Vijayakumar et al., 2016, Raznahan et al., 2011). Gyrification has been found to increase after birth (Kim et al., 2016b), peaking during infancy and decreasing steadily afterwards. Gyrification thus also followed an inverted U-shaped curve (Raznahan et al., 2011). Although large primary and secondary sulci form throughout pregnancy, formation and modification of tertiary sulci continued after birth and throughout childhood (Kim et al., 2016b, Lohmann et al., 1999).
To again ensure comparable results between analyses and to correct for both linear and non-linear effects of age brain structure, age (in months) as well as squared age was included in the GLM as covariates.

2.3.7 Correction for Multiple Comparisons by Threshold Free Cluster Enhancement and Family-Wise Error Rate

As mentioned in 1.2.4, voxel-wise statistical testing includes a great number of statistical tests which also harbors the danger of yielding a high number of false-positive results. Therefore, correction for multiple comparisons is necessary. In this work two approaches were used to this effect, namely the

“Threshold Free Cluster Enhancement” (TFCE) and an additional correction via the “Family-Wise Error Rate” (FWE) correction.

The TFCE toolbox avoids setting a single threshold on all clusters. The algorithm takes into account both the voxel-wise statistical T-value of a given voxel, as well as the size of the cluster it belongs to. The TFCE toolbox maintains the local maximum of a cluster and enhances a clusters’ intensity to facilitate the selection of an appropriate threshold. For each cluster, a range of possible thresholds is applied, and the fitting one across varying thresholds is selected by permutation. Permutation, a randomization test, considers possible correlations between clusters. The algorithm exchanges variables and analyzes the influence of variables on resulting clusters, as well as clusters’ persistency. In this way, correlating clusters can be identified. Additionally, clusters which are persistent in several analyses are assumed to be likely true positives. In contrast to other correcting methods, TFCE corrects data for non-stationarity as cluster sizes depend on the smoothing applied to the image. In general, high smoothness results in larger clusters while low smoothness results in smaller clusters, but this effect is not uniform across the image volume but depends on the local smoothness of the data. It could be shown that TFCE increased sensitivity while effectively controlling type-1-errors (Salimi-Khorshidi et al., 2011, Smith and Nichols, 2009, Pernet, 2016, Li et al., 2016). In all our analyses using this approach, 5000 permutations were calculated for each contrast.

To further correct for false-positive results, FWE-correction on a cluster level (FWE_C) of $p<0.05$ was applied. FWE_C-correction calculates the probability of family-wise errors on a cluster level and sets an initial voxel-threshold above which clusters are assumed to be truly positive results. In this way false-positive clusters are excluded from results. (Nichols and Hayasaka, 2003)

2.3.8 Surface-Based Morphometry

Group differences in data of surface-based morphometry were investigated again using the framework of the GLM. Calculations were conducted in CAT12. As described above, analyses were corrected for gender (described in 2.3.5), linear and non-linear effects of age (described in 0) and multiple comparisons (described in 2.3.7) by TFCE and FWE_C ($p<0.05$).

3 Results

3.1 Participants

We initially approached 94 preterm born children born between July of 1995 and October of 1999 by mail. 44 children could be recruited of which four had MR contraindications (dental braces, metal splinter). Hence, MR images of 40 children could be acquired, among them 23 hCMV-seronegative and 17 hCMV-seropositive, with a median age of 14.8 years (standard error of the mean (SEM) 0.2). Control group participants were recruited by public announcement. They were required to have no history of neonatal infection, no signs of neurological impairment and no MR contraindications. For the control group, images of 43 children, with a median age of 12.0 years (SEM 0.4), were acquired. Consequently, images of 83 children were initially included. Following quality control by automated approaches and manual visual screening, 8 images were excluded, due to bad quality and/or movement artefacts. Consequently, data of 75 images was included in the following analyses: 38 FT and 37 PT, of which 23 belonged to the PT $_{hCMV-}$ and 14 belonged to the PT $_{hCMV+}$ group. Of the 23 PT $_{hCMV-}$, 10 mothers were hCMV-seronegative, while 13 mothers were hCMV-seropositive and did, thus, not transmit hCMV infection to their infants. All mothers of the PT $_{hCMV+}$ were seropositive. There were 42 boys and 33 girls, and median age was 13.6 years (range 7.9-17.8 years; SEM 0.3).

3.1.1 Demographic and Neuropsychological Details of Participants

Demographic data and details of neuropsychologic assessment are shown in Table 1 and Table 2. IQ was determined by the HAWIK IV test and handedness by the EHI. Data was analyzed using Chi-squared tests and Mann-Whitney U tests. Significance was assumed at $p<0.05$. Results were corrected for multiple comparisons by Bonferroni-correction.

Groups FT and PT showed significant differences in age, ME and IQ, but not in gender, handedness and IQR.

Groups PT $_{hCMV-}$ and PT $_{hCMV+}$ did not differ significantly in gender, age, handedness, ME, IQ, IQR, number of twins, gestational age, birth weight, or occurrence of ICH, NEC, BPD and ROP.

	FT	PT	Statistics
n	38	37	
Gender	m: 16 ; f: 22	m: 26 ; f: 11	n.s. [1]
Median age (range) [years]	12.1 (7.9-17.8)	14.9 (12.0-16.1)	p<0.001 [2]
Handedness	87% r ; 13% l	81% r ; 19% l	n.s. [1]
Median ME	15	13	p=0.003 [2]
Median IQ (range)	110 (91-128)	97 (42-137)	p<0.001 [2]
Median IQR (range)	2.44 (2.08-4.05)	2.39 (2.08-3.64)	n.s. [2]

Table 1: Demographic and neuropsychological details of full-term (FT) versus preterm born (PT) participants.

Groups FT and PT differed significantly in age, maternal education (ME) and intelligence quotient (IQ). *P*-values of significant results are given. Differences in gender, handedness and image quality (IQR = image quality rating) was not significant (n.s.). ([1]Chi-squared-test; [2]Mann-Whitney U test. Significance was assumed at *p<0.05*, Bonferroni-corrected.) FT=full-term born participants, PT=preterm born participants; n=number; m=male, f=female; n.s.=not significant; r=right-handed, l=left-handed; ME=maternal education; IQ=intelligence quotient; IQR=image quality rating.

	PT $_{hCMV-}$	PT $_{hCMV+}$	Statistics
n	23	14	
Gender	m: 16 ; f: 7	m: 10 ; f: 4	n.s. [1]
Median age (range) [years]	14.9 (12.1-16.1)	14.8 (12.0-16.1)	n.s. [2]
Handedness	83% r ; 17% l	79% r ; 21% l	n.s. [1]
Median ME	13	13	n.s. [2]
Median IQ (range)	100 (42-137)	93 (61-119)	n.s. [2]
Median IQR (range)	2.39 (2.15-3.64)	2.45 (2.08-3.04)	n.s. [2]
Twins n (%)	7 (30.4%)	7 (50%)	n.s. [1]
Median gestational age (range) [weeks]	28.1 (24-32)	29.7 (25-32)	n.s. [2]
Median birth weight (range) [g]	970 (650-1550)	1272 (630-1870)	n.s. [2]
ICH n (%)	5 (21.7%)	2 (14.3%)	n.s. [1]
NEC n	0	0	
BPD n (%)	5 (21.7%)	1 (7.1%)	n.s. [1]
ROP n (%)	10 (43.5%)	3 (21.4%)	n.s. [1]

Table 2: Demographic and neuropsychological details of preterm born participants with early postnatal hCMV infection (PT $_{hCMV+}$) versus preterm born participants without early postnatal hCMV infection (PT $_{hCMV-}$).
Groups PT $_{hCMV-}$ and PT $_{hCMV+}$ did not differ significantly in demographic, neuropsychological and clinical details. ([1]Chi-squared-test; [2]Mann-Whitney U test. Significance was assumed at $p<0.05$, Bonferroni-corrected.) PT $_{hCMV-}$=preterm born participants without early postnatal hCMV infection, PT $_{hCMV+}$=preterm born participants with earls-postnatal hCMV infection; n=number; m=male, f=female; n.s.=not significant; r=right-handed, l=left-handed; ME=maternal education;IQ=intelligence quotient; IQR=image quality rating (image quality); g=grams; ICH=intracranial hemorrhage; NEC=necrotizing enterocolitis; BPD=bronchopulmonary dysplasia; ROP=retinopathy of prematurity.

To summarize, PT were significantly older at scanning, showed significantly lower ME, and scored significantly lower in IQ testing than FT. Groups PT $_{hCMV+}$ and PT $_{hCMV-}$ showed no significant differences in these parameters, nor were there any significant differences in any of the relevant neonatal risk factors between

two preterm groups. While PT $_{hCMV+}$ showed a tendency towards lower IQ scores than PT $_{hCMV-}$, this difference did not reach significance.

3.1.2 Demographic Details of the Whole Original Cohort of PT

A total of 94 PT were initially approached who had taken part in the original studies on early postnatal hCMV transmission via breastmilk. Of this original cohort, 37 PT were included in the present paper. Of non-participating PT, 24 PT did not answer requests concerning follow-up studies. Further, of PT who had answered requests, six did not have time, one suffered from claustrophobia, eight held magnetic implants and 15 did not give any reasons for their refusal. Data of eight participants had to be excluded due to movement artefacts (see 2.2.6). In order to rule out a selection bias and to make sure that the 37 included PT were representative of the originally available cohort, we compared their demographic details. Details of the groups are listed in Table 3.

Demographic data was analyzed performing Chi-squared tests and Mann-Whitney U tests. Significance was assumed at $p<0.05$. Results were corrected for multiple comparisons by Bonferroni correction.

When comparing included PT with non-participating PT, no differences were found concerning percentage of babies infected with hCMV postnatally, gender, gestational age, birth weight, number of twins, or occurrence of ICH, NEC, BPD and ROP.

	Included PT	Non-participating PT of the original cohort	Statistics
n	37	57	
hCMV+ n (%)	14 (52.6%)	30 (37.8%)	n.s. [1]
Gender	m: 26 ; f: 11	m: 42 ; f: 15	n.s. [1]
Median gestational age (range) [weeks]	28.9 (23.9-32)	28.3 (23.6-32.1)	n.s. [2]
Median birth weight (range) [g]	1140 (630-1870)	1070 (490-1700)	n.s. [2]
ICH n (%)	7 (18.9%)	9 (15.8%)	n.s. [1]
NEC n (%)	0	3 (5.3%)	n.s. [1]
BPD n (%)	6 (16.2%)	7 (12.3%)	n.s. [1]
ROP n (%)	13 (35.1%)	16 (28.1%)	n.s. [1]
Twins n (%)	14 (37.8%)	13 (22.8%)	n.s. [1]

Table 3: Demographic details of included preterm born participants (PT) and non-participating preterm born subjects (PT) of the original cohort.
Groups of included PT and non-participating PT did not differ significantly in demographic details and clinical data. ([1]Chi-squared-test; [2]Mann-Whitney U test. Significance was assumed at $p<0.05$, Bonferroni-corrected.) PT=preterm born participants; n=number; hCMV+=with early postnatal hCMV infection; n.s.=not significant; m=male, f=female; g=grams; ICH=intracranial hemorrhage; NEC=necrotizing enterocolitis; BPD=bronchopulmonary dysplasia; ROP=retinopathy of prematurity.

To summarize, the 37 PT of the original cohort of 94 PT showed no significant differences in demographic and relevant neonatal clinical variables when compared to non-participating PT.

3.2 Global Brain Volumes

To assess a possible global effect of prematurity, we compared absolute values of TIV, GMV, WMV, and CSFV between groups FT and PT. To assess a possible global effect of early postnatal hCMV infection in PT, we compared groups PT_{hCMV-} and PT_{hCMV+}.

Mann-Whitney U tests were conducted, and significance was assumed at $p<0.05$. Results were corrected for multiple comparisons by Bonferroni correction.

3.2.1 TIV

3.2.1.1 PT versus FT

Comparison of FT and PT showed no significant difference in TIV (Figure 2). While PT had a tendency towards lower TIV than FT; this difference was not significant.

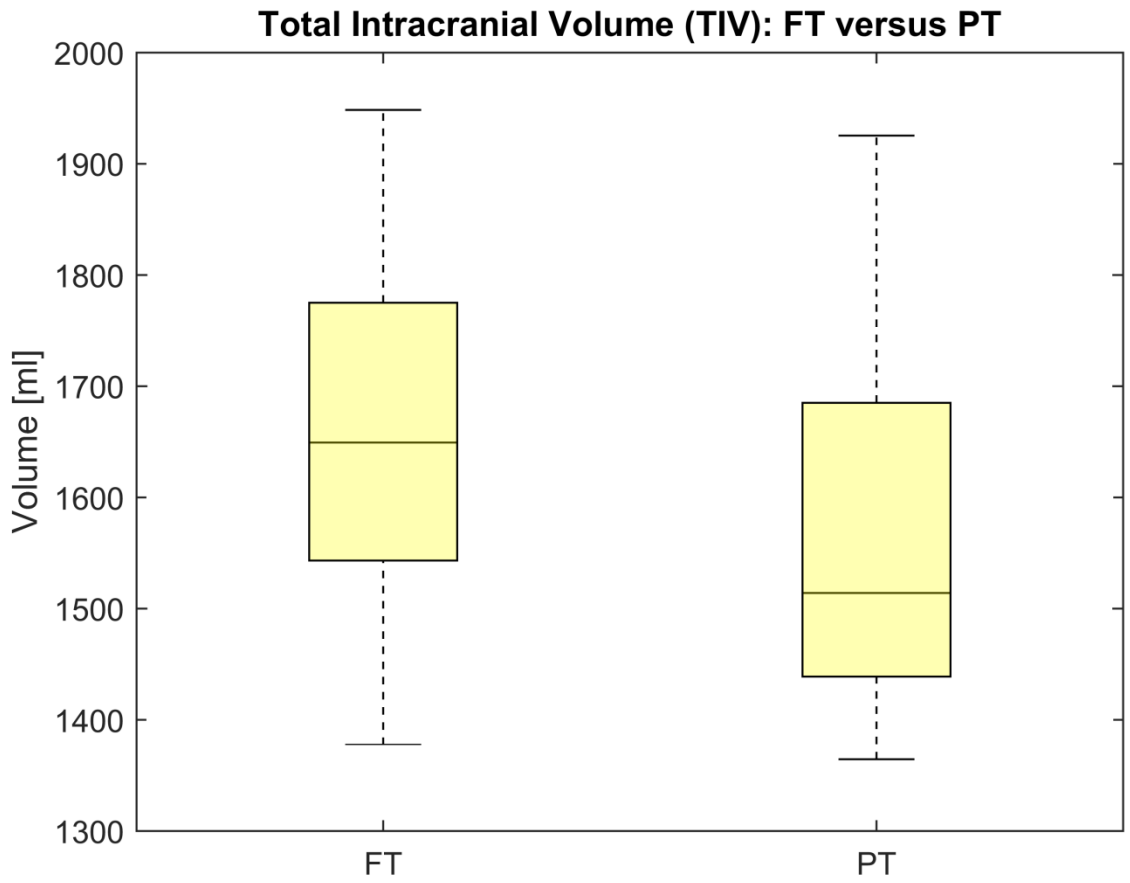

Figure 2: Comparison of total intracranial volume (TIV): full-term (FT) versus preterm born (PT) participants.
Difference in TIV was not significant. (Mann-Whitney U test, significance was assumed at $p<0.05$, Bonferroni-corrected.)

3.2.1.2 PT $_{hCMV+}$ versus PT $_{hCMV-}$

Comparison of PT $_{hCMV+}$ versus PT $_{hCMV-}$ showed no significant difference in TIV (Figure 3). While PT $_{hCMV+}$ had a tendency towards higher TIV than PT $_{hCMV-}$, this difference was not significant.

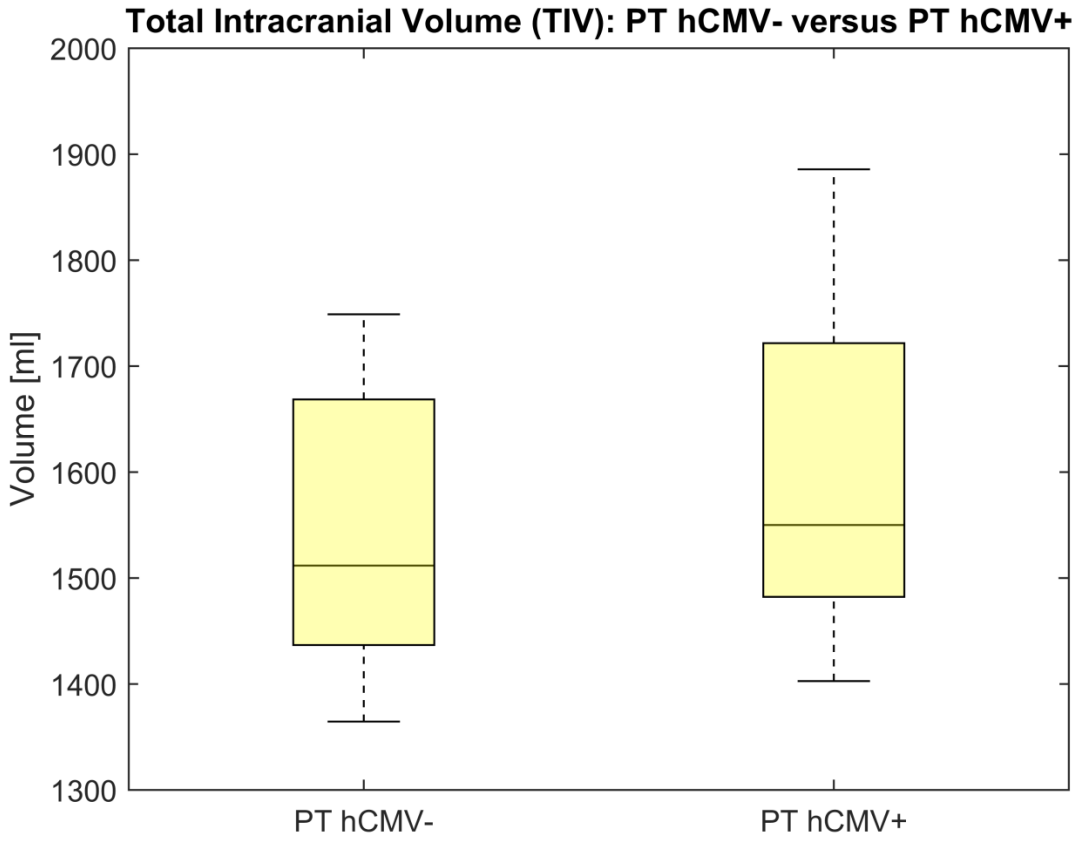

Figure 3: Comparison of total intracranial volume (TIV): preterm born participants without early postnatal hCMV infection (PT $_{hCMV-}$) versus preterm born participants with early postnatal hCMV infection (PT $_{hCMV+}$).
Difference in TIV was not significant. (Mann-Whitney U test, significance was assumed at $p<0.05$, Bonferroni-corrected.)

3.2.2 GMV

3.2.2.1 PT versus FT

PT showed a significant decrease in GMV as compared to FT (Figure 4). In post-hoc targeted comparisons, the difference was driven by the PT $_{hCMV-}$ group which had a significantly lower GMV than FT. In contrast, no significant difference in GMV was found when comparing groups FT to PT $_{hCMV+}$.

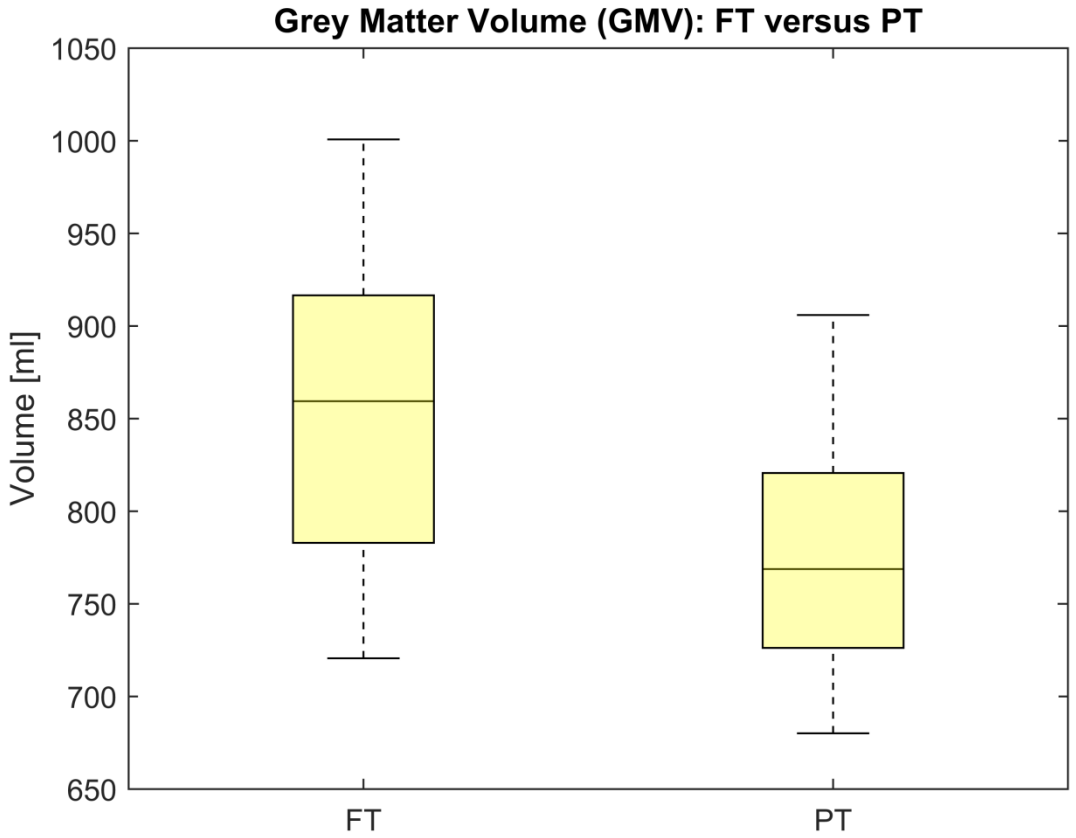

Figure 4: Comparison of grey matter volume (GMV): full-term (FT) versus preterm born (PT) participants.
FT showed a significantly higher GMV compared to PT. (Mann-Whitney U test, significance was assumed at $p<0.05$, Bonferroni-corrected.)

3.2.2.2 PT $_{hCMV+}$ versus PT $_{hCMV-}$

Comparison of PT $_{hCMV-}$ versus PT $_{hCMV+}$ showed no significant difference in GMV (Figure 5). Contrary to our hypothesis, PT $_{hCMV+}$ had a tendency towards higher GMV than PT $_{hCMV-}$, however, this difference was not significant.

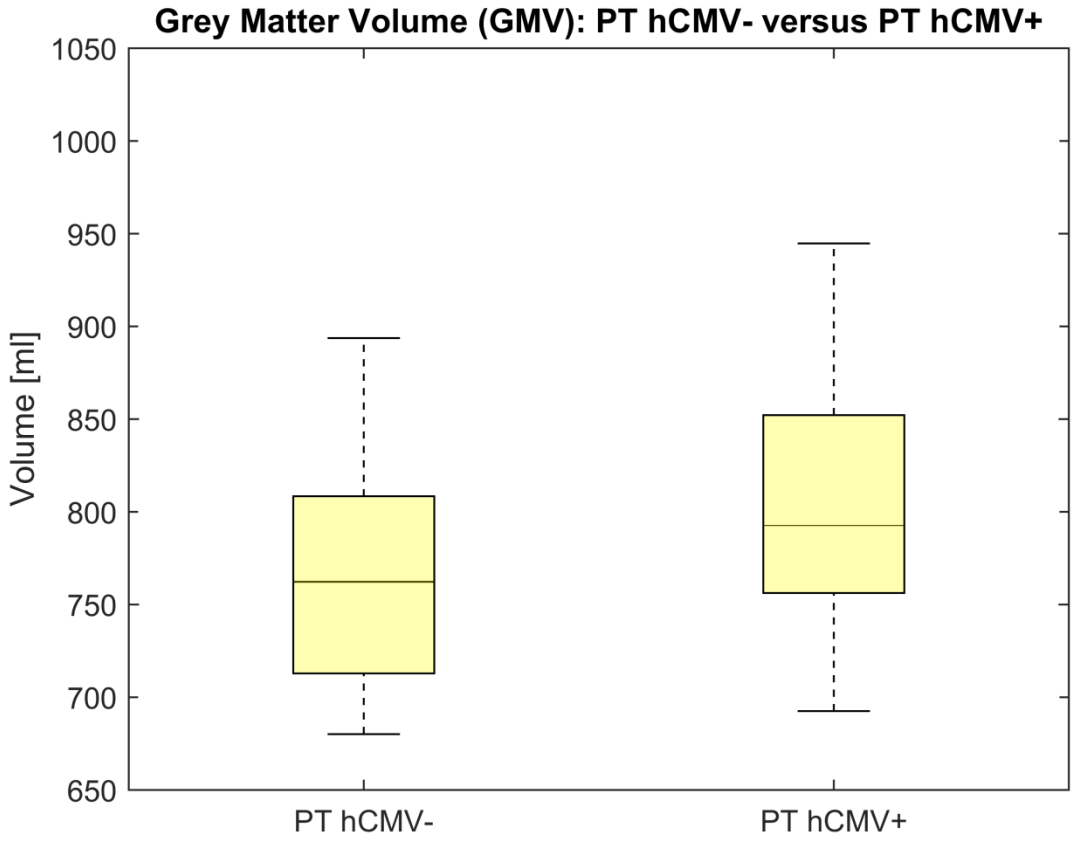

Figure 5: Comparison of grey matter volume (GMV): preterm born participants without early postnatal hCMV infection (PT hCMV-) versus preterm born participants with early postnatal hCMV infection (PT hCMV+).
Difference in GMV was not significant. (Mann-Whitney U test, significance was assumed at $p<0.05$, Bonferroni-corrected.)

3.2.3 WMV

3.2.3.1 PT versus FT

Comparison of PT versus FT showed no significant difference in WMV (Figure 6).

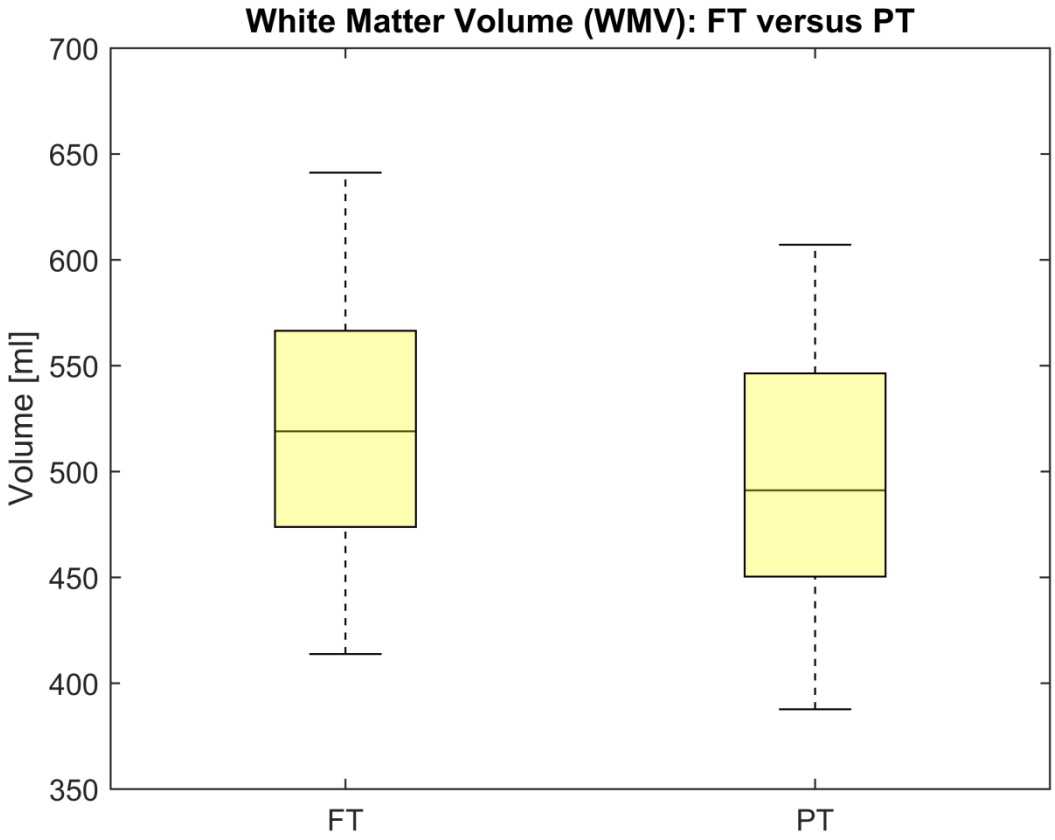

Figure 6: Comparison of white matter volume (WMV): full-term (FT) versus preterm born (PT) participants.
Difference in WMV was not significant. (Mann-Whitney U test, significance was assumed at $p<0.05$, Bonferroni-corrected.)

3.2.3.2 PT $_{hCMV+}$ versus PT $_{hCMV-}$

Comparison of PT $_{hCMV+}$ versus PT $_{hCMV-}$ showed no significant difference in WMV (Figure 7).

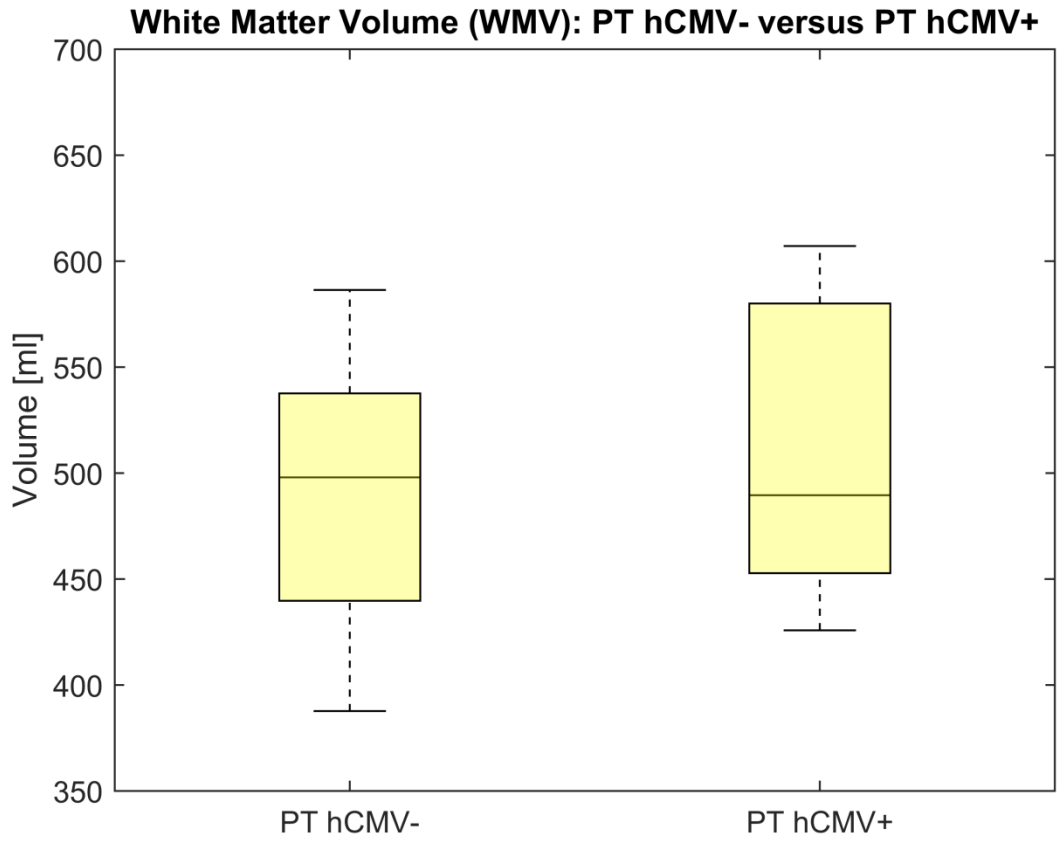

Figure 7: Comparison of white matter volume (WMV): preterm born participants without early postnatal hCMV infection (PT hCMV-) versus preterm born participants with early postnatal hCMV infection (PT hCMV+).
Difference in WMV was not significant. (Mann-Whitney U test, significance was assumed at $p<0.05$, Bonferroni-corrected.)

3.2.4 CSFV

3.2.4.1 PT versus FT

Comparison of PT versus FT showed no significant difference in CSFV (Figure 8).

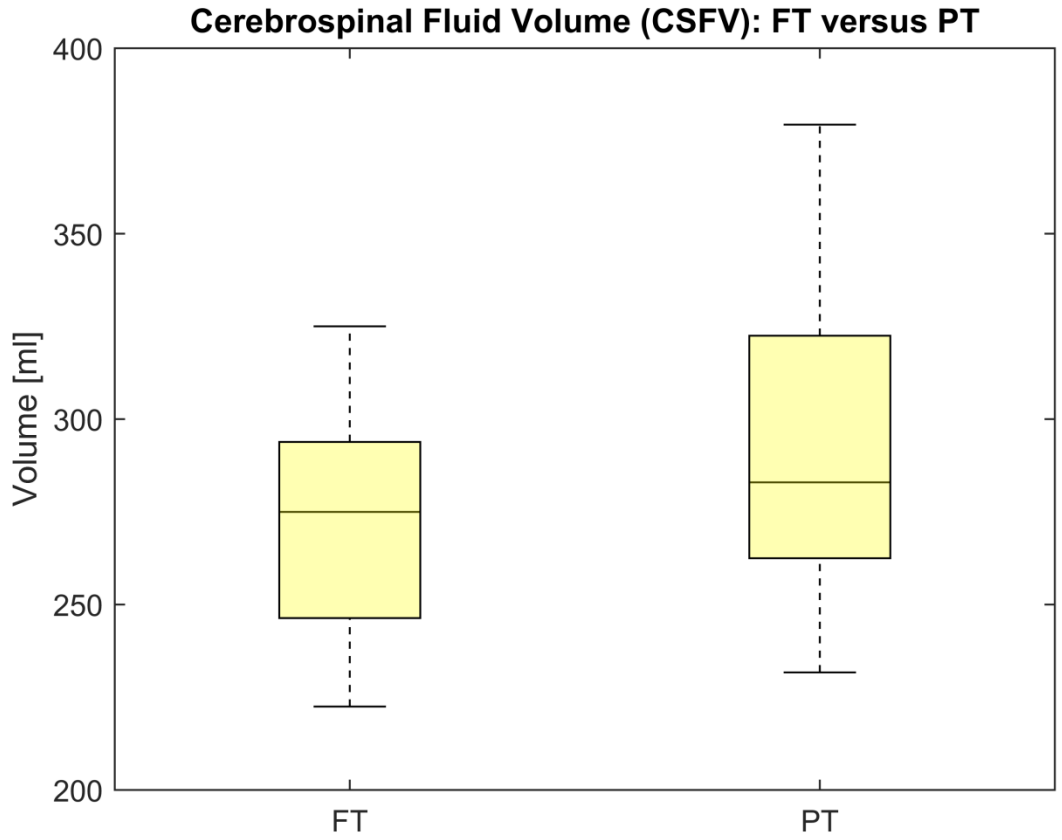

Figure 8: Comparison of cerebrospinal fluid volume (CSFV): full-term (FT) versus preterm born (PT) participants.
Difference in CSFV was not significant. (Mann-Whitney U test, significance was assumed at $p<0.05$, Bonferroni-corrected.)

3.2.4.2 PT $_{hCMV+}$ versus PT $_{hCMV-}$

Comparison of PT $_{hCMV+}$ versus PT $_{hCMV-}$ showed no significant difference in CSFV (Figure 9).

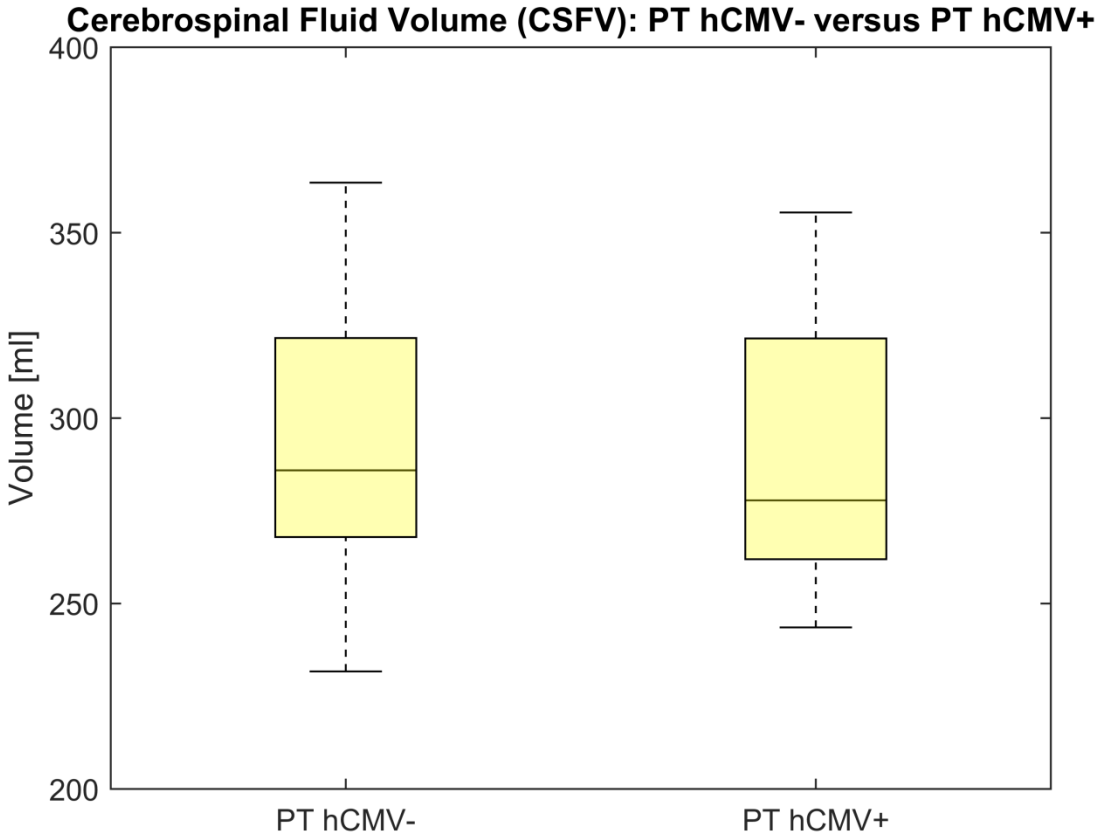

Figure 9: Comparison of cerebrospinal fluid volume (CSFV): preterm born participants without early postnatal hCMV infection (PT hCMV-) versus preterm born participants with early postnatal hCMV infection (PT hCMV+).
Difference in CSFV was not significant. (Mann-Whitney U test, significance was assumed at *p<0.05,* Bonferroni-corrected.)

To summarize results of global brain volumes, GMV was significantly increased in FT compared to PT. Further, FT showed a tendency towards higher TIV than PT, which was, however, not significant. Surprisingly, PT $_{hCMV+}$ did show a tendency, though likewise not significant, towards higher TIV and higher GMV than PT $_{hCMV-}$. WMV and CSFV did not differ significantly between any groups.

3.3 Voxel-Based Morphometry

To assess a possible local effect of prematurity on GMV, we performed VBM studies between groups FT and PT. To assess a possible local effect of early postnatal hCMV infection on GMV in PT, we compared groups PT_{hCMV-} and PT_{hCMV+}.

A GLM was designed, correcting for gender and age (linear and squared) and TIV by global scaling. Significance was assumed after applying TFCE and correcting via FWE_c ($p<0.05$).

3.3.1 Local GMV

3.3.1.1 PT versus FT

PT showed significantly lower local GMV than FT (FT>PT) in the middle temporal gyrus in both hemispheres, in the middle orbitofrontal gyrus in both hemispheres, in the occipital lobe in both hemispheres, in the dorsal temporal lobe in the right hemisphere and in the medial insula in the right hemisphere. Results are visualized in Figure 10. PT showed no significant cluster of increased local GMV, compared to FT (FT<PT).

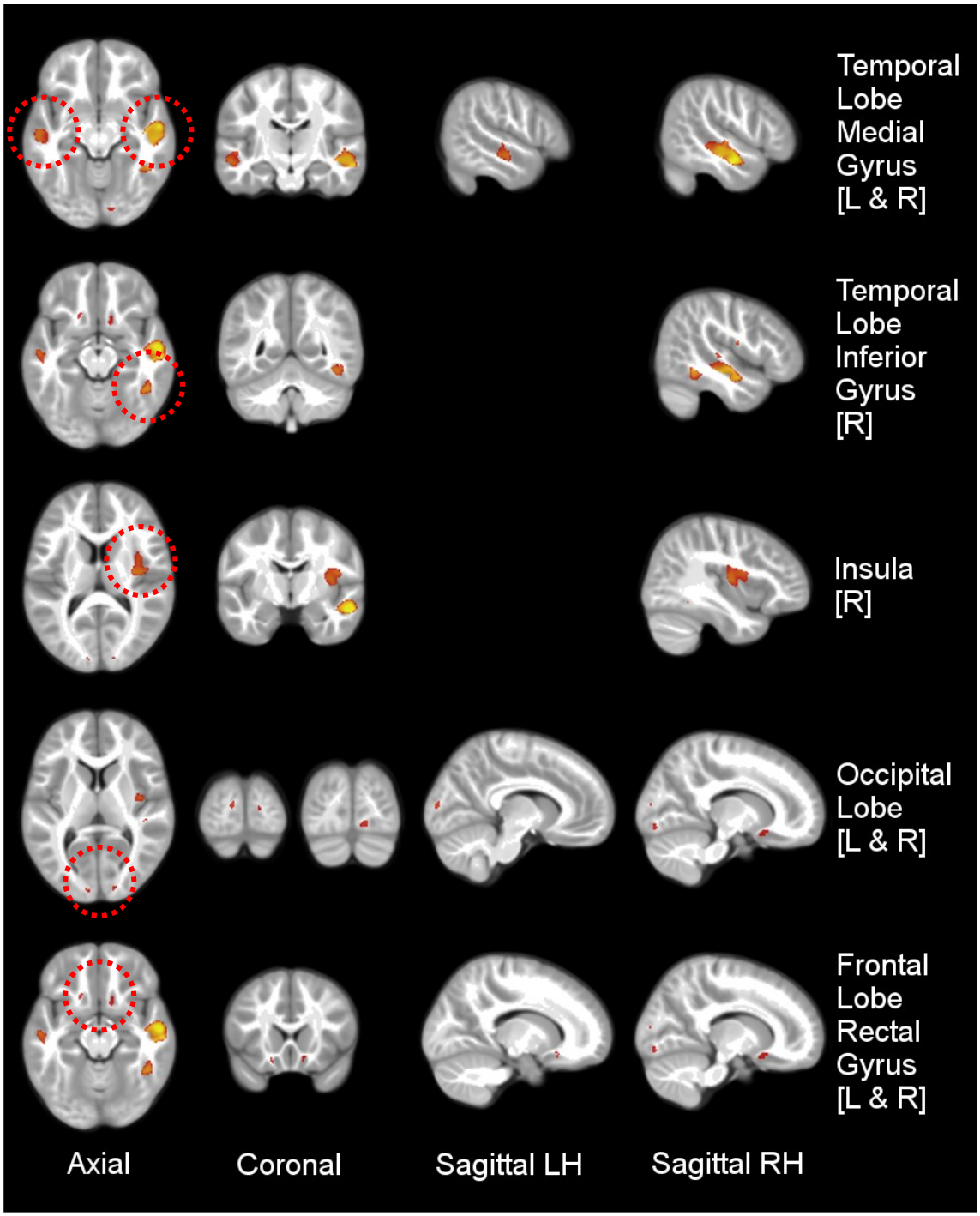

Figure 10: Comparison of local grey matter volume (GMV): full-term (FT) versus preterm (PT) born participants.

PT showed decreased local GMV in the left and right medial temporal lobe, the right dorsal temporal lobe, the left and right occipital lobe, the left and right middle orbitofrontal gyrus and the right insula. Clusters are pointed out by red-dotted circles in the axial view. Clusters are overlaid on the normalized average T1 image of the study group. Results are shown in neurologic convention. (TFCE, FWE $p<0.05$.) LH=left hemisphere, RH=right hemisphere, L=left, R=right.

3.3.1.2 PT $_{hCMV+}$ versus PT $_{hCMV-}$

There were no significant differences in local GMV between PT $_{hCMV+}$ and PT $_{hCMV-}$, in either direction.

To summarize results of local GMV, PT showed areas of significantly lower local GMV than FT. Predominant clusters were localized in temporal lobes and the right insula. Small clusters were localized in the occipital lobe and the frontal gyrus rectus. There were no significant clusters where PT showed a higher local GMV than FT. The comparison of PT $_{hCMV+}$ and PT $_{hCMV-}$ showed no significant difference in local GMV.

3.4 Surface-Based Morphometry

To assess a possible effect of prematurity on cortical surface parameters, we performed analyses assessing cortical thickness, gyrification and sulcal depth for group differences between groups FT and PT. To assess the effect of early postnatal hCMV infection on these parameters in PT, we compared groups PT $_{hCMV-}$ and PT $_{hCMV+}$.

Comparisons were corrected for gender and age (linear and squared). Significance was assumed after applying TFCE and correcting via FWE_c ($p<0.05$).

3.4.1 Cortical Thickness

3.4.1.1 PT versus FT

PT showed locally lower cortical thickness predominantly in the right hemisphere, including temporal (superior, medial and inferior gyrus) and parietal (postcentral, supramarginal and angular gyrus) regions as well as lateral aspects of the occipital lobe, compared to FT. In the left hemisphere, only a small area in the angular gyrus was lower in PT. Results are visualized in Figure 11. There were no significant clusters where PT had higher cortical thickness than FT.

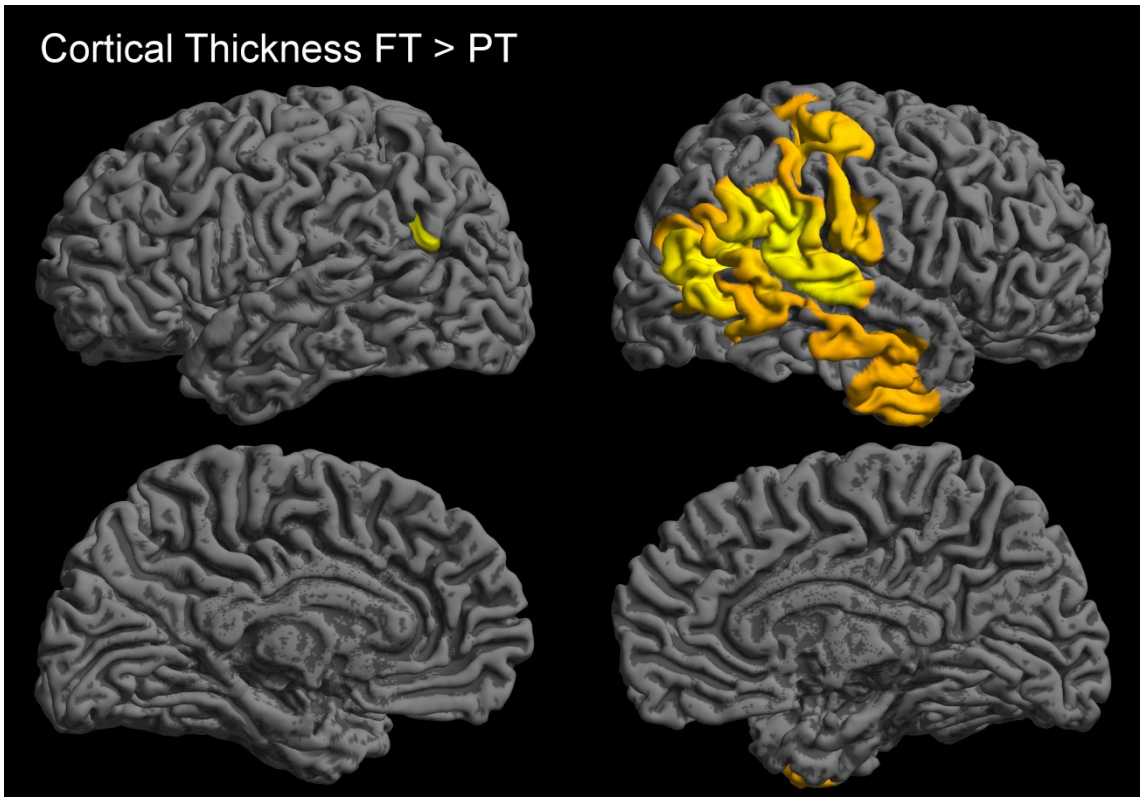

Figure 11: Comparison of local cortical thickness: full-term (FT) versus preterm (PT) born participants.
PT showed significantly lower cortical thickness compared to FT, predominantly in temporal and parietal regions of the right hemisphere. In the left hemisphere, only a small area of parietal lobe showed significantly lower cortical thickness of PT compared to FT. (GLM, TFCE, FWE_c *p<0.05.*)

3.4.1.2 PT $_{hCMV+}$ versus PT $_{hCMV-}$

PT $_{hCMV+}$ showed widespread higher cortical thickness when compared to PT $_{hCMV-}$. Higher cortical thickness was present in all lobes but were more pronounced in the left hemisphere. The left hemisphere showed cluster peaks in the superior and medial temporal gyrus, the frontolateral occipital lobe, the cuneus, pre- and postcentral gyrus, the precuneus and angular gyrus in the parietal lobe, precentral and superior and inferior frontal gyrus, the frontal pole and the cingular gyrus. The right hemisphere showed cluster peaks in the superior and medial temporal lobe, superior parietal lobe, superior frontal gyrus, the frontal pole, and the medial cingular gyrus Results are visualized in Figure 12. There were no clusters of lower cortical thickness in PT $_{hCMV+}$ compared to PT $_{hCMV-}$.

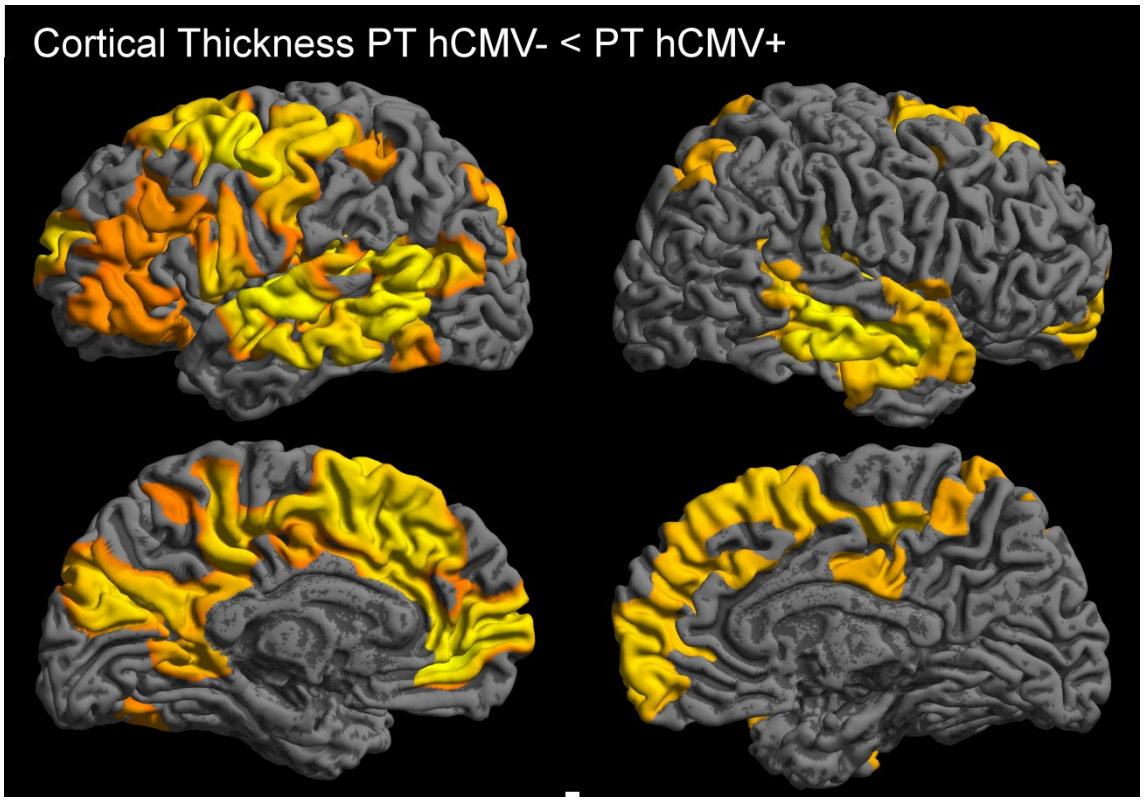

Figure 12: Comparison of local cortical thickness: preterm born participants without early postnatal hCMV infection (PT $_{hCMV-}$) versus preterm born participants with early postnatal hCMV infection (PT $_{hCMV+}$).
PT $_{hCMV+}$ showed widespread significantly higher cortical thickness compared to PT $_{hCMV-}$. This was more pronounced in left hemisphere, including areas of the temporal, parietal, occipital and frontal lobe. In right hemisphere cortical thickness was higher in areas of the temporal, parietal and frontal lobe. (GLM, TFCE, FWE_c $p<0.05$.)

To summarize results of cortical thickness, PT showed higher cortical thickness predominantly in the right temporal and parietal lobe, compared to FT. PT $_{hCMV+}$ showed widespread and bilateral higher cortical thickness when compared to PT $_{hCMV-}$.

3.4.2 Gyrification

3.4.2.1 PT versus FT

PT showed higher values in gyrification which were present in all lobes and slightly more pronounced in the right hemisphere. Cluster peaks were most dominant in the temporal lobe, the occipital pole and the supramarginal gyrus. Results are visualized in Figure 13. There were no clusters where FT showed more gyrification than PT.

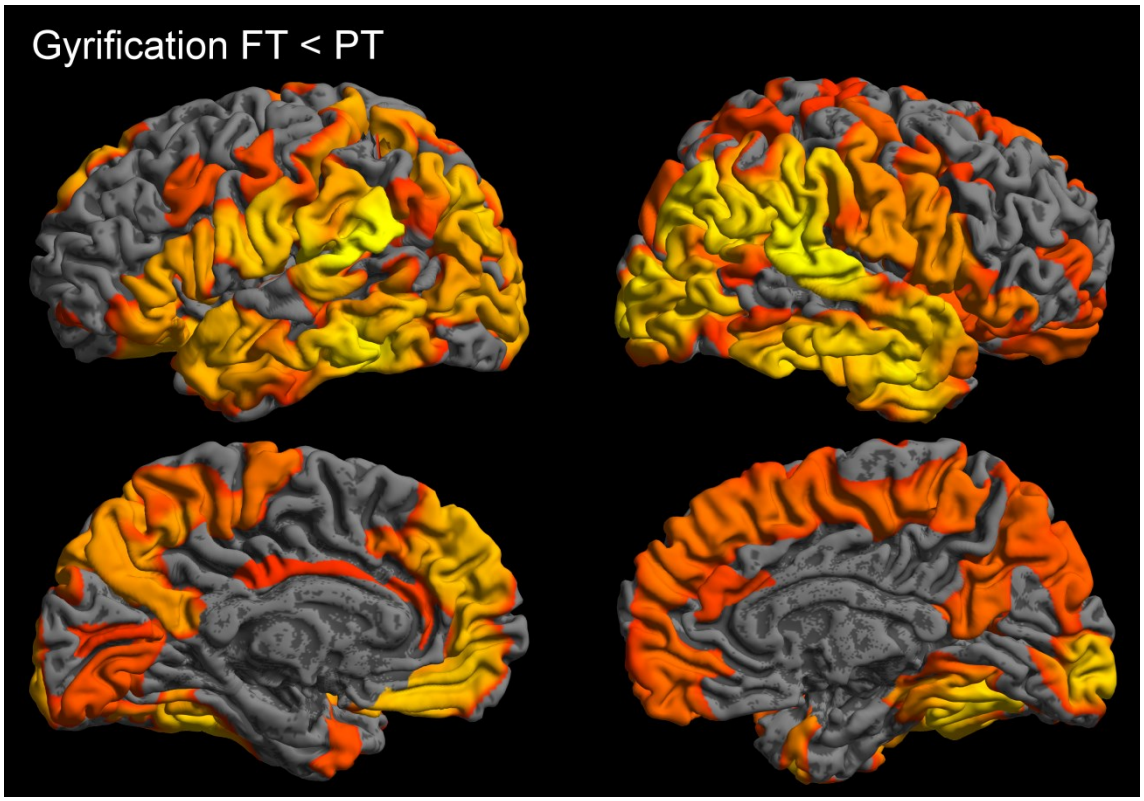

Figure 13: Comparison of local gyrification: full-term (FT) versus preterm (PT) born participants**.** PT showed significantly more gyrification in all lobes of both hemispheres compared to FT. Difference in gyrification peaked in temporal lobes, occipital lobes, gyrus supramarginalis and angularis of parietal lobes. (GLM, TFCE, FWE_c *p<0.05.*)

3.4.2.2 PT $_{hCMV+}$ versus PT $_{hCMV-}$

PT $_{hCMV+}$ showed less gyrification only in very circumscribed regions in the left hemisphere. Small significant clusters were located in the precentral gyrus and in the middle frontal gyrus. Results are visualized in Figure 14. There were no clusters where PT $_{hCMV+}$ showed more gyrification.

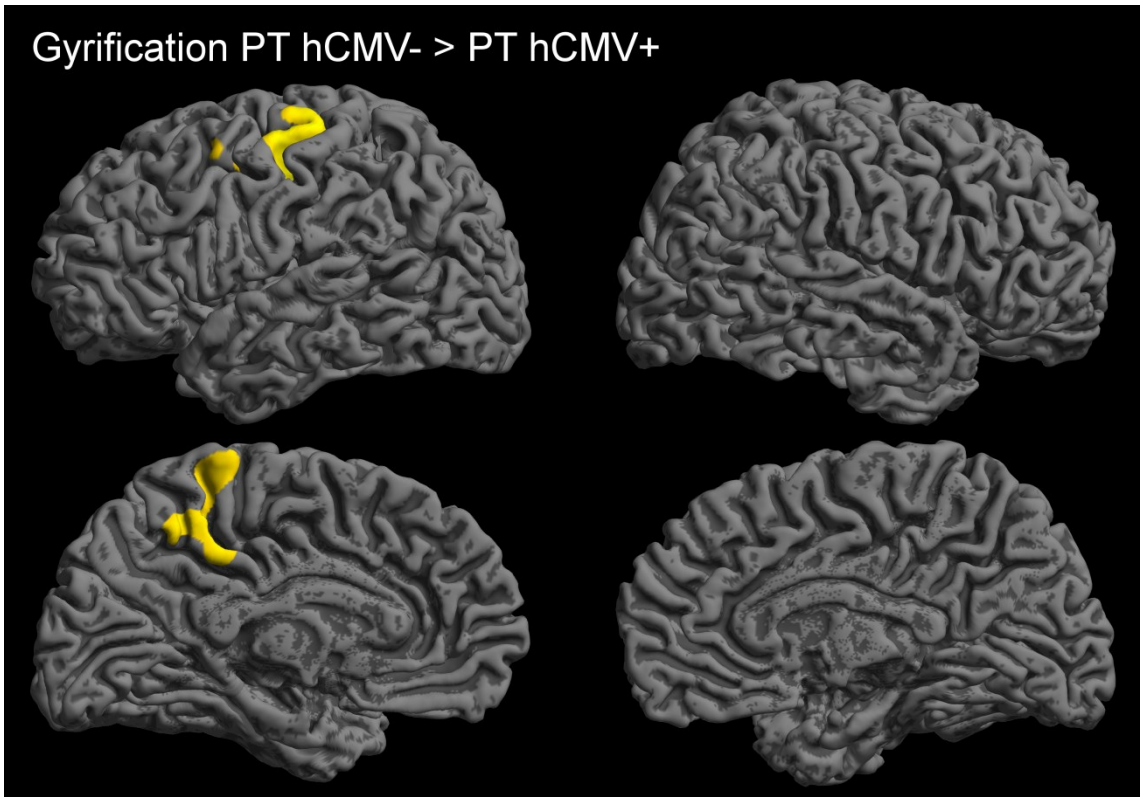

Figure 14: Comparison of local gyrification: preterm born participants without early postnatal hCMV infection (PT $_{hCMV-}$) versus preterm born participants with early postnatal hCMV infection (PT $_{hCMV+}$)**.**
PT $_{hCMV+}$ showed significantly less gyrification in small areas of the left hemisphere compared to PT $_{hCMV-}$. Gyrification of PT $_{hCMV+}$ was lower in gyrus precentralis of the frontal lobe, medial frontal gyrus and gyrus paracentralis. The right hemisphere showed no significant cluster. (GLM, TFCE, FWE_c *p<0.05.*)

To summarize results of gyrification, PT showed widespread and bilateral more gyrification compared to FT. PT $_{hCMV+}$ showed lower gyrification only in two small clusters in the prefrontal gyrus and the paracentral gyrus compared to PT $_{hCMV-}$.

3.4.3 Sulcal Depth

3.4.3.1 PT versus FT

PT showed lower sulcal depth compared to FT in all lobes of both hemispheres, with a slight leftward dominance. Cluster peaks were most dominant in the left and right Sylvian fissure and adjoining parietal and frontal areas. Results are visualized in Figure 15. There were no clusters where PT showed more sulcal depth compared to FT.

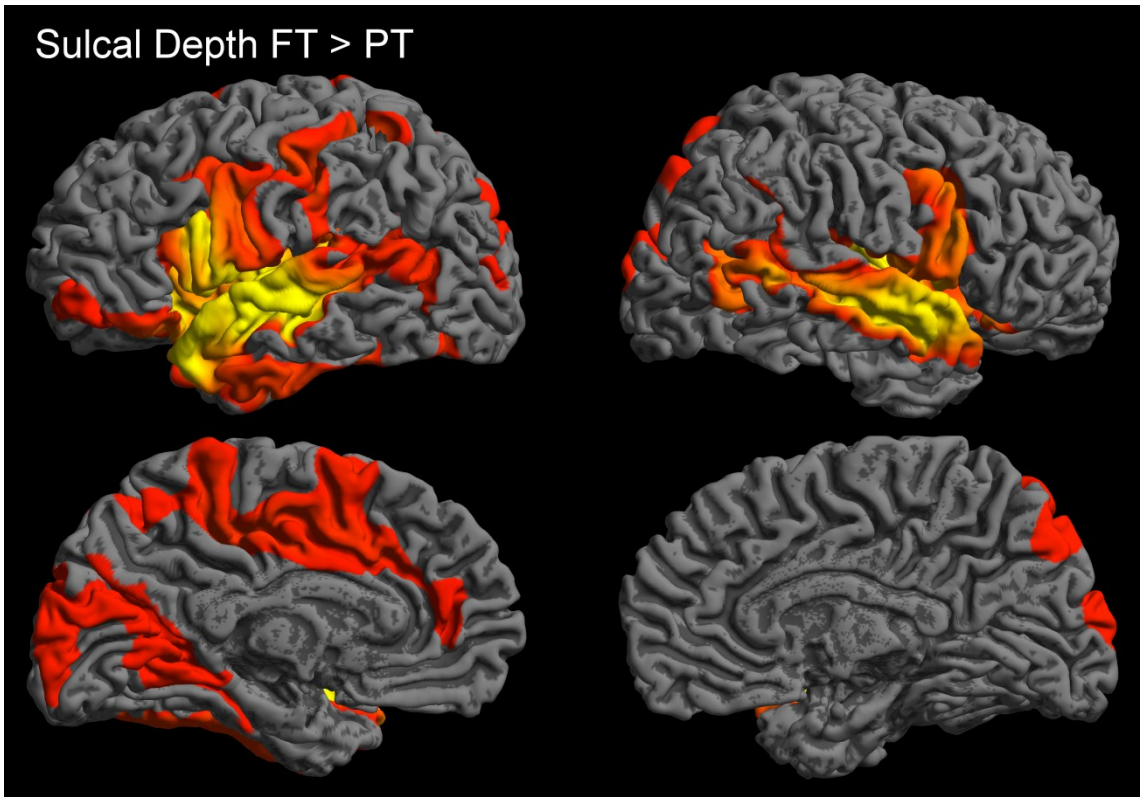

Figure 15: Comparison of local sulcal depth: full-term (FT) versus preterm (PT) born participants**.** PT showed significantly less sulcal depth, compared to FT. Differences peaked in the left and right Sylvian fissure and adjoining sulci of frontal, parietal and temporal lobe. Differences in sulcal depth were slightly more pronounced in the left hemisphere. (GLM, TFCE, FWE_c *p<0.05.*)

3.4.3.2 PT $_{hCMV+}$ versus PT $_{hCMV-}$

There were no significant differences in sulcal depth between PT $_{hCMV-}$ and PT $_{hCMV+}$ in either direction.

To summarize results of sulcal depth, PT showed lower sulcal depth predominantly in the left and right Sylvian fissure and adjoining areas, compared to FT. PT $_{hCMV+}$ and PT $_{hCMV-}$ did not show any significant differences in sulcal depth.

4 Discussion

The aim of this work was to analyze the long-term influence of prematurity in general and of early postnatal hCMV infection in particular, on the brain structure of former preterm born children. To address the first question, all preterm born participants (PT) were compared to all full-term born participants (FT). To address the second question, preterm born children having suffered an early postnatal infection with hCMV (PT_{hCMV+}) were compared to those preterm born children without such an infection (PT_{hCMV-}). Several analyses were conducted. In a first step, global tissue volumes of GM, WM, and CSF were analyzed. Thereafter, local tissue volumes were analyzed by means of voxel-based morphometry. Finally, surface-based morphometry was conducted, assessing cortical thickness, gyrification, and sulcal depth.

Data of 75 participants was included in the analyses, stemming from 38 FT and 37 PT, which included 23 PT_{hCMV-} and 14 PT_{hCMV+}. Median age at scanning of all participants was 13.6 years (SEM 0.3).

We hypothesized that early postnatal hCMV infection would have a long-term consequences on brain structure, above and beyond the influence of prematurity per se.

4.1 Main Findings

4.1.1 PT versus FT

Long-term effects of prematurity on brain structure were investigated by comparing FT to both PT groups. Preceding analysis of demographic and neuropsychological data showed that PT were significantly older at scanning, showed significantly lower maternal education (ME), and scored significantly lower in IQ testing than FT. MRI analyses of global brain volumes showed a tendency of lower TIV in PT than FT, which, however, was only significant when comparing FT to PT_{hCMV-}. PT showed significantly lower global GMV than FT. VBM analyses revealed that local GMV of PT was lower predominantly in temporal lobes and the right insula. Main findings of surface-based analyses showed widespread clusters of higher gyrification in PT, predominantly in temporal lobes, the supramarginal gyrus and the occipital pole. Cortical thickness

was lower in PT in temporal and parietal areas mainly in the right hemisphere. Furthermore, sulcal depth was lower in PT predominantly in the Sylvian fissure bilaterally and in adjoining brain regions.

4.1.2 PT $_{hCMV+}$ versus PT $_{hCMV-}$

Long-term effects of early postnatal hCMV infection on brain structure were investigated by comparing PT $_{hCMV-}$ to PT $_{hCMV+}$. Testing for group differences in demographic, neuropsychologic and clinical data showed no significant differences between groups PT $_{hCMV+}$ and PT $_{hCMV-}$. Interestingly, PT $_{hCMV+}$ scored lower in IQ testing than PT $_{hCMV-}$, which, however, was not significant.

In analyses of global brain volumes, PT $_{hCMV+}$ showed a tendency towards higher TIV compared to PT $_{hCMV-}$. This tendency was more pronounced, though still not significant, when analyzing solely GMV. Analyses of local GMV via VBM showed no significant differences between PT $_{hCMV+}$ and PT $_{hCMV-}$. Our main finding in analyses of surface-based parameters was widespread clusters of higher cortical thickness in PT $_{hCMV+}$, predominantly in temporal and parietal lobes and frontal poles of both hemispheres, with a left-dominance. PT $_{hCMV+}$ showed small clusters of lower gyrification in precentral and paracentral left hemisphere compared to PT $_{hCMV-}$. PT $_{hCMV+}$ and PT $_{hCMV-}$ did not differ significantly in sulcal depth.

4.2 Effects of Prematurity

4.2.1 Impaired Neurocognitive Abilities

There is a large body of evidence regarding cognitive impairment of former PT (Bhutta et al., 2002, Raju et al., 2017, Eryigit Madzwamuse et al., 2015, Hutchinson et al., 2013, Anderson, 2014). In line with the literature, our sample of PT scored significantly lower in IQ testing than FT controls. However, with an average of 97 points, PT still scored within normal range (85-115 points). Interpretation of IQ testing in our sample is, however, limited by the fact that ME was significantly lower in PT than in FT. Maternal education is known to play an important role in the neurocognitive outcome after prematurity (Patra et al., 2016), as well as being linked to prematurity per se (Ruiz et al., 2015). Hence, our results

are well in line with previous studies on neurocognitive impairment of preterm born children at school-age and beyond.

4.2.2 Reduced Global GMV

Results of our global analyses showed significantly lower GMV in PT, compared to FT. Those results were in accordance with previous studies, showing lower GMV at term-equivalent age as well as later on (Monson et al., 2016, Thompson et al., 2007, Padilla et al., 2015). Scanning at term-equivalent age further showed decreases in WMV and presumably compensatory increase in CSFV (Thompson et al., 2007). As in the full-term born control group, WMV and GMV of preterm born children increased throughout childhood, but WMV and GMV of preterm born children did not catch up with the volumes of the full-term born group till the age of 7. The difference in GMV between preterm born and full-term born children even increased throughout childhood (Monson et al., 2016). In our sample there was no significant effect of prematurity on WMV and CSFV. Discrepancies may have resulted from a higher rate of ICH, WM injury and neonatal complications in other published preterm born groups (Monson et al., 2016, Thompson et al., 2007). Reduced GMV in infancy and childhood was associated with lower scores in IQ testing in the literature (Monson et al., 2016), which is in line with our observations.

4.2.3 Reduced Local GMV

Reduced GMV in the middle temporal gyrus, as was apparent in our PT group compared to FT, has been reported various times in groups with a history of premature birth across a wide age range (nine-year-olds (Soria-Pastor et al., 2009, Zubiaurre-Elorza et al., 2011), male 12-year-olds (Kesler et al., 2008), adolescents (Nosarti et al., 2008) and adults (Bauml et al., 2015).

Furthermore, our analyses showed lower local GMV in right dorsal inferior temporal gyrus in PT compared to FT. Lower GMV in the inferior temporal gyrus has been reported previously in preterm born young adults; interestingly, also only in the right hemisphere in both studies (Nosarti et al., 2014, Bauml et al., 2015). Likewise regional brain volume measurement in preterm born infants at

term-equivalent age revealed lower volume of the right inferior temporal gyrus (Gousias et al., 2012).

In our study, other analyses of brain structure revealed alterations in the temporal lobe as well (see below), suggesting a particular vulnerability of the temporal lobe to preterm birth. Temporal lobe alterations will be collectively discussed in 4.2.7.

Moreover, the insula of the right hemisphere showed lower local GMV in VBM analyses. Lower GMV in insular regions were reported previously in adolescents (Nosarti et al., 2008) and in young adults (Nosarti et al., 2014) with a history of preterm birth. Our results showed a cluster only in the right hemisphere. A study examining adolescents and young adults with additional learning support, however, described association of reduced GMV in the *left* insula with low birthweight (Spencer et al., 2008). The Insula functions as an integrating center of various networks. As part of the limbic system, it plays a role in sensory and motoric networks, distinguishes between salient and irrelevant impulses from internal and external stimuli, influences attention, focuses working memory and plays a role in human consciousness (Menon and Uddin, 2010, Augustine, 1996, Craig, 2009). The right insula seems to play a greater role in survival-related stimuli, like pain-related internal stimuli, learning and self-recognition, whereas the left insula may have a greater role in emotional and positive stimuli. (Craig, 2009) It remains unclear, in which ways lower GMV in the left insula is functionally relevant. However, impairment of stimulus control and distinguishing of salient stimuli is often part of the behavioral phenotype frequently described in preterm born individuals (Delobel-Ayoub et al., 2006, Treyvaud et al., 2013). Interestingly, results of sulcal depth analyses also showed a cluster located, among other regions, bilaterally in the insula. It seems possible that insula is likewise particularly vulnerable to the adverse consequences of preterm birth. (Results of sulcal depth analyses are further discussed in 4.2.6.)

Analyses of local GMV further revealed lower GMV in bilateral middle orbitofrontal cortex in PT compared to FT. Reduced GMV in the middle orbitofrontal cortex has been described previously in preterm born individuals at term-equivalent age (Ball, 2012). Further, very preterm born adults showed less depth of secondary sulci in orbitofrontal areas (Gimenez et al., 2006). The middle orbitofrontal cortex

was associated with decision making and solving of conflicts. It has been reported that patients with damage to the orbital prefrontal cortex had difficulties in gambling tasks and in choices between actions that differed in probability, punishments and rewards (Rogers et al., 1999). Patients with lesions in the ventromedial prefrontal cortex, containing the cluster of interest, had difficulties in planning ahead, in choosing friends, partners and activities and in overcoming temptations (Bechara, 2004). A group of adolescents with ADHD showed impaired processing of reward prediction errors, with aberrances in fMRI of the orbital prefrontal cortex (Hauser et al., 2014). While these may be behavioral manifestations of the structural alterations observed here, the ultimate clinical relevance of structural alterations of the middle orbitofrontal cortex is yet unclear. Lastly, PT compared to FT showed lower GMV in small clusters located in the occipital lobe. Reduced GMV in the occipital cortex has already been described in preterm born adolescents (Nosarti et al., 2008) and young adults (Nosarti et al., 2014). The clusters, though small, are located in the extrastriate visual cortex. The extrastriate visual cortex plays a role in object, face and number recognition, spatial attention and visuospatial information (Desimone, 1998, Allison et al., 1994, Kesner et al., 1993). Visuospatial problems are widespread in former preterm born individuals, especially in connection with posterior WM lesions (Pavlova et al., 2006, Pavlova et al., 2007, Pavlova et al., 2003). These problems may be enhanced by additional GM lesions within the same system, but no analyses were done here to correlate possible visuospatial impairments in our subjects with these volume reductions in the extrastriate cortex.

4.2.4 Lower Cortical Thickness

Cortical thickness was predominantly lower in PT in the superior and middle temporal lobe, interestingly only in the right hemisphere. Lower cortical thickness in the temporal lobe has already been reported in 15 and 20-year-old teenagers with low birth weight (Bjuland et al., 2013, Martinussen et al., 2005), in the left temporal lobe of 7-12-year-old preterm born children (Murner-Lavanchy et al., 2014), in bilateral middle and inferior temporal lobe of 15-year-old preterm born individuals (Nagy et al., 2011) and in 16-year-old preterm born teenagers (Frye et al., 2010). Bjuland and colleagues further reported an association of lower

cortical thickness in the temporal lobe with perceptual organization index of IQ testing, though not full-scale IQ (Bjuland et al., 2013).

Local analyses of GMV likewise indicated greater impairment of the right temporal lobe. Since receptive language functions are predominantly located in the left hemisphere, the predominance of alterations in the right hemisphere is puzzling and the clinical relevance of our results' asymmetry remains unclear.

4.2.5 Higher Gyrification

Our analyses showed higher gyrification in PT compared to FT in all lobes of both hemispheres. Investigations of previous studies reported divergent results of higher (Kesler et al., 2006) or lower (Zhang et al., 2015) gyrification in preterm born school-aged children. Increase in gyrification, as widespread as was seen in our results, suggests that prematurity had an impact on a process that affected the whole brain. Since gyrification was found to decrease in healthy children from about six years of age (Raznahan et al., 2011), higher gyrification may be a sign of delayed cortical maturation in preterm born individuals. Delay in cortical maturation of the preterm brain has been described previously. Estimations of brain age based on structural MR images, compared to the participants' true chronological ages, showed greater discrepancies between estimated and true age in preterm born, than in full-term born individuals (Franke et al., 2012). Furthermore, previous studies reported delayed cortical thinning in preterm born preschool children (Phillips et al., 2011), school-aged children (Murner-Lavanchy et al., 2014) and adolescents (Nam et al., 2015, Klein et al., 2014), which implied delayed cortical maturation as well. Our PT sample did not show strong evidence of delayed cortical thinning, since there were no brain regions where PT had higher cortical thickness than FT. Even so, in temporal-occipital areas cortical thickness was also found to increase in adolescents (Vijayakumar et al., 2016), hence, higher cortical thickness in temporal lobe would not entirely rule out delay in cortical maturation.

The widespread and robust higher values in gyrification in the PT group suggests that prematurity had an impact on a process that affected almost the whole brain, sparing only the latest-maturing frontal poles on both sides. We suggest that higher gyrification in PT might be the detectable structural correlate of delayed

whole-brain maturation of PT. Peaks of higher gyrification in PT, among others in temporal areas, would support our suggestion that the temporal lobe is particularly vulnerable to complications associated with preterm birth.

Gyrification is the result of a complex folding process designed to increase cortical surface such that more grey matter can be fit into the limited cavity of the human skull (Albert and Huttner, 2015). According to Volpe's theory of brain damage in preterm born children (Volpe, 2009), described in detail in 1.2.10, cortical brain alterations are a consequence of early WM damage in the weeks following premature birth. Subsequently impaired cortical development postnatally may, in the long-term, lead to the findings observed here. Therefore, the foci of higher gyrification in the PT group could reflect a long-term consequence of an early white matter damage.

Alternatively, however, they could be the result of a compensatory mechanism to make up for this early interference with brain development. In healthy children, higher gyrification was usually associated with *higher* cognitive abilities (Gregory et al., 2016, Gautam et al., 2015) which is clearly not the case in our PT sample. Our results could therefore also be interpreted as being a late consequence of an early disruptive event in so far as that, postnatally, higher gyrification may be a mechanism to make up for the early negative impact. In order to test this hypothesis, however, serial MRI measurements and developmental testing would be needed to assess when the increases of gyrification develop postnatally and what their functional correlate is.

4.2.6 Lower Sulcal Depth

Sulcal depth was lower in PT compared to FT, predominantly in the bilateral Sylvian fissure and adjoining areas. Previous studies reported similar findings: In 7-year-old former preterm born children, lower sulcal depth in anterior parts of the superior temporal lobe was found (Zhang et al., 2015). In another study investigating sulcal depth of preterm born children directly after birth, preterm born children showed impaired cortical folding till term-equivalent age in, among others, right middle temporal lobe (Kim et al., 2016a). Intrauterine MRI showed that primary sulci were not yet fully developed till 33 weeks of gestation. Superior temporal sulcus evolved at 26 to 30 weeks of gestation. (Garel et al., 2001) Thus,

formation of even large temporal sulci is still ongoing when preterm born children are born, and impairment, as indicated by our results, seems possible. Even though other sulci were likewise still evolving, our results suggest that they do not seem to be affected as severely as temporal and parietal sulci, which adjoin Sylvian fissure.

4.2.7 Summary of the Pattern of Structural Brain Alterations in Our Sample of PT as a Function of Prematurity

Several of our analyses show the temporal lobe to be predominantly affected in our sample, either bilaterally (analyses of local GM, gyrification, sulcal depth) or on the right side only (analysis of cortical thickness).

In a premature baboon model, injury of the temporal lobe's middle and superior gyrus was one of the most commonly described brain damages (Dieni et al., 2004). It has further been suggested that the temporal lobe is particularly vulnerable to preterm birth because synaptogenesis increased rapidly in the temporal lobe exactly in those weeks when preterm born children are born. The temporal lobe was also described to be particularly vulnerable to hypoxic injuries (Kesler et al., 2006). We therefore suggest that our results are in line with findings indicating enhanced vulnerability of the temporal lobe in preterm born children.

Of the same cohort of preterm born children as analyzed in this paper, data on altered functional connectivity of superior temporal lobes in PT compared to FT has been published previously. In PT, the connectivity between both superior temporal lobes was decreased, whereas connectivity to other regions of the brain was increased. The authors suggested that impaired interhemispheric connectivity resulted from WM lesions, whereas increased connectivity to other brain regions indicated compensating connections (Wilke et al., 2014). This work was in part motivated by, and did ultimately independently confirm, a previous study on reduced structural connectivity between the two temporal lobes in former preterm born children (Northam 2012). These previous findings may be associated with our current findings of lower local GMV in temporal lobes and may therefore indicate that structural alterations correlate with functional impairment. The temporal lobe is known to play a role in language processing (Helenius et al., 2014, Marslen-Wilson and Tyler, 1980), reading acquisition

(Monzalvo and Dehaene-Lambertz, 2013), working memory (Jeneson and Squire, 2012) and learning (Dalton et al., 2016). Further, studies have previously reported an association of altered temporal lobe structure with cognitive abilities. In preterm born young adults, lower GMV in medial temporal lobe was associated with lower executive function scores (Nosarti et al., 2014). In children with dyslexia, lower GMV was found in posterior areas of temporal lobe (Eckert et al., 2016), which match cluster peaks in our analyses of gyrification and cortical thickness, though not findings in local GMV analyses. Lower GMV in inferior temporal gyrus has likewise been found in low birth weight school children requiring learning support (Kesler et al., 2008). Hence, structural alterations of the temporal lobe may play a role in impairment of cognitive, reading, speech and learning abilities, all of which have been described in preterm born children (Bowen et al., 2002, Hutchinson et al., 2013, Aarnoudse-Moens et al., 2011).

4.3 Effects of Early Postnatal hCMV Infection in PT

The main objective of this work was to analyze the consequences of early postnatal hCMV infection on brain structure of preterm born children. We hypothesized that hCMV would have a long-term consequence on brain structure, above and beyond the influence of prematurity.

4.3.1 Neurocognitive Abilities of PT $_{hCMV+}$

More in-depth analyses of the neuropsychological data of PT, originating from the same original cohort (Bevot et al., 2012, Brecht et al., 2015, Goelz et al., 2013), have already been published. At the medium age of eight, comparison of PT $_{hCMV+}$ and PT $_{hCMV-}$ revealed significant cognitive and motor impairment of PT $_{hCMV+}$. Test scores of PT $_{hCMV+}$ were at the lower end, but still within the normal range. Still, PT $_{hCMV+}$ more often required special learning support. (Bevot et al., 2012) Regarding cognitive skills at an average age of 13.6 years, we could previously show in a slightly larger group that only PT $_{hCMV+}$ had significantly lower IQ values than FT. No significant difference was found when comparing FT to PT $_{hCMV-}$, suggesting that cognitive impairment of the whole PT group was mainly driven by PT $_{hCMV+}$ (Brecht et al., 2015). In the cohort of the present work, significant differences between PT $_{hCMV+}$ and PT $_{hCMV-}$ in IQ testing could not be replicated.

Though PT $_{hCMV+}$ showed a tendency towards lower IQ scores (median IQ PT $_{hCMV+:}$ 93 vs. PT $_{hCMV-}$: 100), differences did not reach significance. This is explained by data of three PT $_{hCMV+}$ which had to be excluded in this work due to movement artefacts in the MRI data. The different composition and the smaller sample size likely explain these discrepancies.

4.3.2 Global Brain Volumes of PT $_{hCMV+}$

As expected, analyses of global brain volumes revealed that PT $_{hCMV+}$ showed a tendency, though not significant, towards lower TIV and GMV compared to FT controls. Surprisingly though, compared to PT $_{hCMV-}$, PT $_{hCMV+}$ showed a tendency towards greater TIV and GMV, although also not reaching significance. Counter-intuitively, difference in global volumes was therefore less pronounced between PT $_{hCMV+}$ and FT than between PT $_{hCMV-}$ and FT. Cortical volume is defined as product of cortical thickness and surface area (Wierenga et al., 2014, Raznahan et al., 2011). Of those two parameters, cortical thickness was calculated directly in this paper. Surface area, in contrast, was not directly analyzed. However, surface area is influenced by gyrification, which defines the ratio of visible to hidden brain surfaces. Gyrification differed only slightly between PT $_{hCMV-}$ and PT $_{hCMV+}$. Thus, gyrification did most likely not influence global GMV substantially. Gyrification of PT $_{hCMV-}$ and PT $_{hCMV+}$ will be discussed further in 4.3.5. However, PT $_{hCMV+}$ showed higher cortical thickness in widespread areas of the brain, which is further discussed in 4.3.4. We therefore suggest that the widespread thicker cortical in PT $_{hCMV+}$ compared to PT $_{hCMV-}$ is the mechanism that results in more global GMV in PT $_{hCMV+}$. Thus, the counter-intuitive higher volume of GM in PT $_{hCMV+}$ would indirectly indicate local structural alterations in cortical thickness.

4.3.3 hCMV Infection Did Not Influence Local GMV

Analyses of local GMV did not yield any significant clusters when comparing PT $_{hCMV-}$ to PT $_{hCMV+}$. Since various authors have reported influence of prematurity on local GMV, as described in 1.2.8, an effect of hCMV infection on local GMV seemed likely. However, results showed no significant differences as a function of hCMV infection on local GMV in our cohort. It was previously suggested that

local GMV, cortical surface and cortical thickness are the product of at least partly independent processes during brain development (Wierenga et al., 2014). Since effects of early postnatal hCMV infection on brain structure showed a completely different pattern of brain alterations than effects of prematurity on brain structure, it could be concluded that different underlying processes were affected, but at this point, this is mere speculation.

4.3.4 Higher Cortical Thickness in PT $_{hCMV+}$

The main finding of our analyses investigating the influence of early postnatal hCMV infection on brain structure was a widespread thicker cortex in PT $_{hCMV+}$ compared to PT $_{hCMV-}$. Thus, it can be assumed that early postnatal hCMV infection in PT affected various parts of the brains which still resulted in higher cortical thickness at the age of assessment (~13.6 years). Since PT $_{hCMV+}$ were compared to PT $_{hCMV-}$, these differences were no consequence of prematurity per se. As described above, many other neonatal risk factors were also balanced between the two groups, suggesting that differences can be specifically attributed to the postnatal hCMV infection

Higher cortical thickness in late childhood may indicate primary long-term aberrations in formation of cortical columns as a function of hCMV infection, which then persisted throughout childhood till the date of our measurements (and probably beyond). However, they could also represent secondary alterations, reflecting a delay in brain maturation. These ”primary and secondary damage” hypotheses will be discussed in detail in 4.3.6 and 4.3.7, respectively.

4.3.5 Small Clusters of Less Gyrification in PT $_{hCMV+}$ and no Significant Differences in Sulcal Depth

Analyses of surface data showed only two small clusters of less gyrification in PT $_{hCMV+}$, compared to PT $_{hCMV-}$, in the left postcentral and paracentral gyrus. A mechanical model of cortical folding posits that higher cortical thickness leads to a stiffening of the cortical surface, leading to less cortical folding and less gyrification (Budday et al., 2014, Gautam et al., 2015). The local clusters of less gyrification could thus be a result of the higher cortical thickness in PT $_{hCMV+}$ in those areas. However, the latter are very widespread, while only small areas

showed less gyrification. While these could reflect “the tip of the iceberg”, we did not perform focused post-hoc analyses to assess this hypothesis in a more direct way.

Analyses of sulcal depth did not show any significant difference between PT $_{hCMV+}$ and PT $_{hCMV-}$. Combined, gyrification and sulcal depth determine cortical surface area (Raznahan et al., 2011). Cortical surface area and cortical thickness were suggested to be at least partly driven by different evolutionary processes, as well as different genes (Rakic, 1995, Jansen and Andermann, 2005). Thus, alteration of gyrification and sulcal depth, as opposed to alteration of cortical thickness, may reflect the impairment of different processes. Whereas cortical surface is determined by the number of neural columns within the cortex, cortical thickness is determined by the number of neurons and neuropil within those columns. The number of columns within the cortex mainly depends on the amount of postmitotic neural cells, which are generated in the subventricular zone and migrated successfully into the cortex. Therefore, mitotic activity in the subventricular zone would determine cortical surface. Since a greater number of neurons within one column leads to a thicker cortex, cortical thickness also would depend on mitotic activity. But apart from that, efficiency of arrangement within the columns and the amount of interneurons and neuropil also have an influence on the thickness of cortical columns (Rakic, 1995). Intracortical organization mainly occurs after cell proliferation and migration and is affected by different processes, such as myelination and formation of long-distance cortical connections (Barkovich et al., 2001).

Summarizing the surface-based analyses assessing the effect of early postnatal hCMV infection, our results show that cortical thickness was more affected than gyrification and sulcal depth. This may reflect a predominant disorder of columnar organization within the cortex. Previous research has shown that development of cortical thickness, at least in the first year of life, was determined by cortical thickness at birth (Meng et al., 2017), indicating that cortical thickness was a sensitive parameter for early cortical alterations in the form of disturbed intra-cortical organization. This would explain the dominant effect on cortical

thickness despite no significant effect on local GMV and sulcal depth, and only minor effects on gyrification.

Our results further showed greatly differing impacts of prematurity and early postnatal hCMV infection. These findings suggest that those two pathologies lead to different patterns of cortical injury. Further, only by combining the different imaging parameters analyzed in this paper were our analyses sensitive to these different underlying patterns of cortical alterations. In order to give a detailed description of brain alteration and avoid missing structural alterations, analysis of several structural parameters can therefore be confirmed to be advantageous (Wierenga et al., 2014).

4.3.6 Potential Primary Brain Damage in Early Postnatal hCMV Infection of PT

Early postnatal hCMV infection in preterm born infants chronologically equates congenital hCMV infection in the third trimester. In contrast to infections in the first and second trimester of pregnancy, which can cause microcephaly, lissencephaly and polymicrogyria, infections in the third trimester were traditionally described to have no macroscopic influence on brain morphology (Barkovich and Lindan, 1994). Moreover, it has been described that intrauterine hCMV infections in third trimester were not associated with long-term sequelae (Foulon et al., 2008, Oosterom et al., 2015, Enders et al., 2011). However and as already mentioned, PT with early postnatal hCMV infection showed cognitive impairment (Bevot et al., 2012, Brecht et al., 2015) in previous studies. Compared to fetuses, preterm born infants additionally must cope with strenuous extrauterine environment and immaturity-related comorbidities. Furthermore, quantitatively the largest amounts of maternal antibodies are transmitted via the placenta during the third trimester (Simister, 2003, Saso and Kampmann, 2020). Therefore, preterm born infants lack passive maternal immunoprotection. For all those reasons, it seems likely that preterm born infants are at a higher risk of an adverse outcome from early postnatal hCMV infection as compared with fetuses suffering from a congenital hCMV infection in the third trimester, although they were principally of the same age.

As described in 1.3.3, congenital hCMV infection can induce structural brain abnormalities depending on the time of infection. Whereas early congenital

infection resulted in schizencephaly and lissencephaly, mid-gestational infection may lead to polymicrogyria (Barkovich and Lindan, 1994). Further, focal cortical dysplasia, which is sometimes difficult to detect in MR images (Kim et al., 2011), has not only been associated with human papilloma virus infection (Chen et al., 2012), but also with hCMV infection (Liu et al., 2014). Brain alterations in congenital hCMV infection as described above show the severe impact hCMV can have on cortical organization.

In MRI of our PT $_{hCMV+}$-group, no overt brain abnormalities as sometimes seen in congenital hCMV infection (Picone et al., 2014) were found. However, our findings suggest less obvious brain alterations. Those seem plausible, regarding the obvious detrimental potential of hCMV in congenital infection.

The pathogenesis of early postnatal hCMV infection on a microscopic level has not yet been investigated. In contrast, hCMV's impact in congenital infection is well-described. In congenital hCMV infection, the four main pathomechanisms which are said to disrupt brain development are, firstly, predominant damage of stem cells (Teissier et al., 2014), secondly, impeded cell migration into the cortex (Cheeran et al., 2009), thirdly, susceptibility of glial cells to hCMV infection (Cheeran et al., 2009) and, fourthly, immune response per se (Gabrielli et al., 2012). When discussing these in early postnatal hCMV infection, some potential pathomechanisms arise.

Since firstly, the main body of stem cells has already differentiated in preterm born infants and, secondly, cellular migration into the cortex is mostly complete at 24 weeks of gestation, the relevance of first two pathomechanisms in postnatal hCMV infection is doubtful. Nevertheless, a still-present population of progenitor cells plays a role in formation of thalamocortical and association fibers. And further, within the cortex, neurons still organize, forming local and long-distance synapses. (Volpe, 2009)

More crucial could be, thirdly, the damage of glial cells in early postnatal hCMV infection, since axon formation is still under way in the third trimester (Kostovic and Judas, 2002). It may be speculated that impeded axon development may also have an impact on cortical structure.

Fourthly, the harmful effect of inflammatory processes per se on preterm born infants was shown by a study stating that systemic inflammation was one of the two determining factors of cognitive abilities in former preterm born toddlers (Leviton et al., 2013). Cytokines and chemokines secreted in inflammatory processes change the micro-environment in the developing brain and were described to possibly alter neural differentiation and migration throughout congenital hCMV infection (Cheeran et al., 2009). Those may also interfere with cortical organization in the preterm brain.

In conclusion, primary brain damage in early postnatal hCMV infection has not yet been investigated in enough detail. Extrapolating investigations of congenital hCMV infection, a detrimental potential of hCMV is obvious, whereas it can only be speculated on the exact underlying pathomechanism in early postnatal hCMV infection in preterm born children.

4.3.7 Potential Secondary Impact of Early Postnatal hCMV Infection in PT on Brain Development

As mentioned in 4.3.4, altered brain structure at the age of 13.6 years may either result from primary damage throughout early postnatal hCMV infection, or from secondary sequelae by means of altered brain maturation, or as the result of compensatory mechanisms. Possible neural pathologies of primary brain damage were discussed in the previous paragraph 4.3.6. This paragraph will focus on the possible secondary impact of early postnatal hCMV infection.

Previous research on brain development showed that MR scans at birth could predict cortical thickness development in the first year of life with high accuracy. Those findings indicate that dynamics of cortical thickness development, at least in the first year, were to a great extend determined already at birth. (Meng et al., 2017)

As previously described, cortical thickness decreases in most areas of the brain throughout late childhood and adolescence (Raznahan et al., 2011, Schnack et al., 2015, Vijayakumar et al., 2016, Aleman-Gomez et al., 2013). There is a body of literature suggesting that the here-observed higher cortical thickness in our school-aged sample of preterm born children should not actually be seen as an increase, but rather has to be interpreted as a lack of the naturally-occurring

thinning and thus, as a delay in cortical maturation (Murner-Lavanchy et al., 2014, Nam et al., 2015).

In line with this, pain-derived stress of preterm born babies in NICU primarily affects WM and subcortical GM in the first weeks of life (Brummelte et al., 2012), but leads to lower cortical thickness in former preterm born children at 7 years of age (Ranger et al., 2013). As inflammation related pain was included (Ranger et al., 2013), this could be the common pathogenic mechanism at work.

Moreover, cortical thinning was associated with cognitive abilities in children. The more pronounced cortical thinning was, and the earlier cortical thinning set in, the higher IQ was (Schnack et al., 2015, Shaw et al., 2006). Consistent with this observation, higher cortical thickness in preterm born children was associated with lower IQ at 12 years of age (Brouwer et al., 2014) and lower executive function at 15 years of age (Nam et al., 2015). Hence, a delay in cortical thinning may be associated with impaired cognitive abilities of PT $_{hCMV+}$, which were described in previous publications on this cohort (Bevot et al., 2012, Brecht et al., 2015).

4.3.8 Comparison to Previous Research on Brain Structure of PT $_{hCMV+}$

So far, only few studies have focused on brain structure of preterm born children with early postnatal hCMV infection.

One study performed cranial ultrasound at term-equivalent age. Nijman and colleagues described higher incidence of lenticulostriate vasculopathy in preterm born infants with early postnatal hCMV infection, whereas no higher incidence of IVH could be found (Nijman et al., 2012a). At term-equivalent age, this group of hCMV+ preterm born infants further showed lower fractional anisotropy in occipital WM, indicating microstructural WM alterations (Nijman et al., 2013). In preterm born infants, PVL and subsequent WM damage is said to be the main driving pathology leading to cortical structural alterations (Volpe, 2009). Based on MRI analyses, it has been suggested that congenital hCMV infection showed a similar pattern of white matter affection as does PVL (van der Voorn et al., 2009). These findings would support the theory that in postnatal hCMV infection, at least part of the cortical alterations are due to an underlying WM damage, as suggested before for more typical preterm brain WM lesions (Volpe, 2009).

In an fMRI study on the same study cohort as investigated for this work, PT $_{hCMV+}$ showed higher brain activation in language tasks compared to PT $_{hCMV-}$, suggesting that the same language tasks required a higher cognitive effort in PT $_{hCMV+}$ than in PT $_{hCMV-}$. (Dorn et al., 2014) Those findings indicate that structural cortical alterations in PT $_{hCMV+}$ were indeed associated with functional impairment. In neuropsychological testing, functional impairment of PT $_{hCMV+}$ outside the language domain has been demonstrated (Brecht et al., 2015, Bevot et al., 2012).

In conclusion, previous research on early postnatal hCMV infection in preterm born infants indicates that WM damage could be at least one plausible underlying cause of GM alterations seen in our results. Further, the GM alterations visible in our work may be associated with functional alterations and cognitive impairment.

4.4 Strengths and Limitations of This Work

The first limitation of this paper is relatively small sample sizes. Of 94 PT from the original cohort, only 37 were included in the present thesis. PT not included did not answer requests, did not want to participate, had contraindications for MRI, or showed movement artefacts in their MR data. However, PT included in this thesis did not differ significantly in postnatal clinical data from the whole original cohort and were, thus, presumably representative of the original cohort. Further, of 37 included PT, only 14 were hCMV+. Small sample sizes may not accurately represent the patient cohort they claim to represent. On the other hand, while smaller groups may lack the power to detect subtle differences, the differences that are detected can be taken to be robust (if appropriately controlled for multiple comparisons, as done here).

Whereas serologic an PCR testing in the PT population clearly defined those PT having suffered from an early-postnatal infection with hCMV, there was no data available for the FT control group. Unfortunately, no testing had taken place following their full-term birth. As described in 1.3.4, publications on hCMV transmission via breast milk focused mostly on hCMV transmission in study populations of preterm born infants whereas detailed data on this transmission in *full-term* born children is lacking. Nevertheless, we can assume that, according

to serologic analyses of breast-feeding mothers in Tübingen, about 50% of the mothers were hCMV positive while breast feeding their full-term born infants later included in this study as our control group (Hamprecht and Goelz, 2017). Therefore, we can suspect that at least some of the full-term born children were infected early-postnatally with hCMV via breast milk of their hCMV-positive mothers. The extent of this transmission, however, remains unclear.
Beyond that, there is so far no significant scientific evidence that post-natal hCMV transmission and infection in full-term born children has adverse effects on the affected children's brain development. Still, it cannot be ruled out that such undocumented infections in our full-term control group may have had an impact on the results of our analyses. We may, however, assume that such an impact would have rather diminished the differences between groups that produced false positive results.

Regrettably, significant differences in age between PT and FT made it necessary to correct for differences in age. Nevertheless, PT $_{hCMV-}$ and PT $_{hCMV+}$ did not differ significantly in age. Analyses between PT $_{hCMV-}$ and PT $_{hCMV+}$ therefore cannot be expected to be driven or mitigated by age differences. Moreover, PT were older than FT and, thus, effectively had "a head start" in brain development, and age was further corrected for by including it as a covariate. If anything, it must be expected that significant differences in age might have led to an underestimation of alterations in brain structure between PT and FT.

Furthermore, our cross-sectional data cannot answer the question of possible effects over time. For example, the question whether early postnatal hCMV infection had a primary or secondary impact on brain structure cannot be answered. Analyses of longitudinal MR data, or MR data within the first year of life combined with our findings would be required to this effect.

This work is based on a prospective and single-center study. HCMV infection of PT was precisely diagnosed and clinical data for scientific purposes was available

for all subjects. Neuropsychological assessment as well as MR data acquisition was blinded regarding prematurity and early postnatal hCMV infection.
MR data was preprocessed according to current recommendations, using appropriate pediatric reference data. We also employed current approaches for VBM and surface data analyses. A potential bias by image quality differences was ruled out since groups did not differ significantly in the selected image quality measure.

Due to the now-recognized detrimental effects of early postnatal hCMV infection (Bevot et al., 2012, Brecht et al., 2015, Dorn et al., 2014, Goelz et al., 2013, Hamele et al., 2010, Fischer et al., 2012, Anne-Aurelie et al., 2016), the original study design could not as easily be repeated in Germany. Therefore, our data and results are a unique source of knowledge on the impact of early postnatal hCMV infection on brain structure and neurocognitive abilities in former preterm born children. To the best of our best knowledge, no comparable data on long-term outcome of early postnatal hCMV infection has been published so far.

4.5 Conclusion

The main aim of this paper was to analyze long-term influence of early postnatal hCMV infection on brain structure of preterm born children.
Across several analyses, there were both robust effects of prematurity (differences between FT and PT) and of early postnatal hCMV infection (differences between PT_{hCMV+} and PT_{hCMV-}).
Remarkably, the patterns of cortical alterations were markedly different from each other, with little overlap in the overall pattern. This suggests a distinct and independent impact of either factor.
In agreement with previous studies, our former preterm born participants showed decreased global and local GMV. Those findings were supplemented by differences in surface-based analyses which have not yet been so widely reported in preterm born children. Widespread higher clusters of higher gyrification and local clusters of lower sulcal depth in PT underline the profound impact of prematurity on brain development.

HCMV-infected PT showed widespread clusters of higher cortical thickness compared to non-infected PT, which strongly indicates that early postnatal hCMV infection had a long-term consequence on brain structure independently of the influence of prematurity per se. Counter-intuitive higher global GMV in PT $_{hCMV+}$ could be explained by widespread higher cortical thickness. This effect may reflect impaired cortical maturation due to impaired cortical proliferation and/or organization by early postnatal hCMV infection in our PT sample. Especially considering detrimental effects of intrauterine hCMV infection on cortical organization, this explanation seems plausible.

Since our results demonstrate that early postnatal hCMV infection has a long-term consequence on brain structure of former preterm born children, we strongly support further implementation of efforts to prevent such an infection in preterm born infants.

5 Summary

While congenital, intrauterine infection with human Cytomegalovirus (hCMV) is the most common non-genetic cause of severe neurodevelopmental disability, postnatal infection of a full-term born infant will usually not lead to an adverse neurodevelopmental outcome. In preterm born infants, however, long-term consequences of an early postnatal infection with hCMV are still controversial. Previous neuropsychological and functional MR studies already indicated such additional long-term effects exist.

This thesis investigated long-term consequences of early postnatal hCMV infection on brain structure in former preterm born children as compared to full-term born controls. We hypothesized that such long-term consequences are detectable, independently of the influence of prematurity per se.

Data of 37 preterm born children (PT), born ≤ 32 weeks of gestation and/or weighing ≤ 1500g was included in this work, 14 of which acquired an early postnatal infection with hCMV (PT_{hCMV+}); 23 remained hCMV- (PT_{hCMV-}). Further, 38 healthy full-term born participants (FT) were included. Median age at scanning of all 75 participants (33 girls) was 13.6 years (range 7.9-17.8 years). Demographic and neuropsychological data, as well as global brain volumes, were analyzed by Mann-Whitney U tests or Chi-squared tests as appropriate, assuming significance at $p<0.05$ (Bonferroni-corrected) in SPSS 23 (IBM, Armonk, NY, USA). T1-weighted 3D datasets (TR/TE=1300/2.92ms, resolution 1×1×1 mm^3) were acquired using a 1.5 T Avanto magnetic resonance (MR) scanner (Siemens, Erlangen, Germany). MR data was preprocessed and analyzed in Matlab (R2014b, The Mathworks, Natick, USA) using CAT12 (Gaser & Dahnke, Jena University, Germany), a toolbox running within SPM12 (Wellcome Trust Centre for Neuroimaging, University College London, UK), using pediatric reference data. A general linear model was designed, correcting for global volumes via “global scaling”. Gender and age were included as covariates of no interest, and multiple comparisons were accounted for via threshold-free cluster enhancement (TFCE) and family-wise error rate correction (FWE_C; $p<0.05$). Local grey matter volume (GMV) was analyzed using voxel-based

morphometry (VBM). We also analyzed parameters describing the cortical surface, including cortical thickness, gyrification, and sulcal depth.

In order to assess the consequence of prematurity, FT were compared to PT. PT scored significantly lower in IQ testing. As expected, PT showed significantly lower *global* GMV as well as several areas of lower *local* GMV, compared to FT. The main finding of the surface analyses between PT and FT was a widespread clusters of higher gyrification in PT.

In order to assess the consequence of early postnatal hCMV infection, PT $_{hCMV-}$ were compared to PT $_{hCMV+}$. PT $_{hCMV+}$ had a tendency towards lower IQ scores, which, however, was not significant. Interestingly, PT $_{hCMV+}$ showed a tendency towards higher global GMV compared to PT $_{hCMV-}$, although this again was not significant. Main finding in the surface analyses were unexpectedly widespread cluster of higher cortical thickness in PT $_{hCMV+}$, compared to PT $_{hCMV-}$. Confirming and extending previous studies, we could show lower global and local GM volumes, as well as lower gyrification, as a function of premature birth. The main focus, however, was to assess the impact of an early postnatal hCMV infection. To this effect, comparison of PT $_{hCMV-}$ to PT $_{hCMV+}$ showed that early postnatal hCMV infection in PT had a long-term influence on brain structure. Remarkably, the patterns of cortical alterations in those analyses were markedly different from those resulting from prematurity per se, suggesting a distinct and independent impact of either factor.

Counter-intuitive higher global GMV in PT $_{hCMV+}$ could be explained by widespread thicker cortex. This effect may reflect impaired cortical maturation due to impaired cortical proliferation and/or organization induced by early postnatal hCMV infection. Especially considering substantial effects of intrauterine hCMV infection on cortical organization, this explanation seems plausible.

To conclude, early postnatal hCMV infection had a long-term influence on brain structure in our sample of former preterm born children. Therefore, efforts to avoid such an infection in preterm born infants should continue to be implemented.

Zusammenfassung

Während eine kongenitale, intrauterine Infektion mit humanem Cytomegalievirus (hCMV) die häufigste nicht-genetische Ursache einer angeborenen, schweren geistigen Behinderung ist, hinterlässt eine postnatale Infektion mit hCMV bei einem reifgeborenen Kind in der Regel keine neurologischen Langzeitfolgen. Bei einem Frühgeborenen sind die langfristigen Auswirkungen einer früh-postnatalen hCMV Infektion noch umstritten. Neuropsychologische und funktionelle MR-Studien zeigen bereits Hinweise auf eine zusätzliche, langfristige Beeinträchtigung. In dieser Arbeit wurden langfristige Auswirkungen einer früh-postnatalen hCMV Infektion auf die Gehirnstruktur bei ehemaligen Frühgeborenen im Vergleich mit reifgeborenen Kontrollen untersucht. Wir stellten die Hypothese auf, dass solche langfristigen Auswirkungen nachweisbar sind, und zwar zusätzlich zum Effekt von Frühgeburtlichkeit an sich.

Untersucht wurden Daten von 37 Frühgeborenen (FG), geboren ≤ 32 Schwangerschaftswochen und/oder mit ≤ 1500g Geburtsgewicht, wovon sich 14 früh postnatal mit hCMV infizierten (FG $_{hCMV+}$), 23 blieben hCMV- (FG $_{hCMV-}$). Außerdem wurden Daten von 38 gesunden Reifgeborenen (RG) untersucht. Das mediane Alter aller 75 Probanden, davon 33 Mädchen, bei der Untersuchung war 13,6 Jahre (7,9-17,8 Jahre). Demographische und neuropsychologische Daten, sowie die globalen Gehirnvolumina wurden mittels Mann-Whitney U Test oder Chi-Quadrat-Test mit einem Signifikanzniveau von $p<0,05$ (Bonferroni-korrigiert) in SPSS 23 (IBM, Armonk, NY, USA) analysiert. Es wurden T1-gewichtete 3D Datensätze (TR/TE=1300/2,92ms, Auflösung 1×1×1mm³) an einem 1,5T Avanto Magnetresonanz (MR)-Tomographen (Siemens, Erlangen, Deutschland) aufgenommen. Die MR Daten wurden innerhalb von Matlab (R2014b, The Mathworks, Natick, USA) mit CAT12 (Gaser & Dahnke, Universität Jena, Deutschland), einer Toolbox in SPM12 (Wellcome Trust Centre for Neuroimaging, University College London, UK) vorverarbeitet. Die Analyse erfolgte auf Basis von pädiatrischen Referenzgewebekarten. Für die Analyse wurde ein Generelles Lineares Modell (GLM) verwendet. Mittels „global scaling“ wurde für globale Volumenunterschiede korrigiert. Geschlecht und Alter wurden als Kovariaten berücksichtigt. Für multiple Vergleiche wurde mittels

„threshold-free cluster enhancement“ (TFCE) und „family-wise error rate“ (FWE_C; $p<0,05$) korrigiert. Analysiert wurden das lokale Volumen an grauer Substanz mittels voxel-basierter Morphometry (VBM), sowie die Oberflächenparameter kortikale Dicke, Gyrierung und Sulcustiefe.

Um die Einflüsse der Frühgeburtlichkeit zu untersuchen, wurden FG mit RG verglichen. Die FG zeigten signifikant niedrigere IQ Werte als die RG. Bei den FG war wie erwartet *global* und *lokal* in einigen Clustern das Volumen an grauer Substanz signifikant niedriger als bei den RG. Als Hauptergebnis der Oberflächenanalysen zeigte sich bei den FG eine großflächig verstärkte Gyrierung.

Der Einfluss einer früh-postnatalen hCMV Infektion bei FG wurde im Vergleich von FG $_{hCMV-}$ vs. FG $_{hCMV+}$ analysiert. FG $_{hCMV+}$ wiesen eine Tendenz zu geringeren IQ-Werte auf, allerdings ohne statistische Signifikanz. Interessanterweise zeigten FG $_{hCMV+}$ vs. FG $_{hCMV-}$ eine Tendenz zu höherem globalen Volumen an grauer Substanz, allerdings ebenfalls ohne statistische Signifikanz. Dahingegen zeigte sich überraschenderweise in den Oberflächenanalysen bei den FG $_{hCMV+}$ eine großflächig und signifikant erhöhte kortikale Dicke gegenüber FG $_{hCMV-}$.

Konkordant und ergänzend zu bisherigen Studien wiesen die FG global und lokal niedrigere Volumina an grauer Substanz sowie großflächig verstärkte Gyrierung auf. Der Fokus dieser Arbeit lag auf dem Vergleich zwischen FG $_{hCMV-}$ und FG $_{hCMV+}$. Hier zeigte sich, dass eine früh-postnatale hCMV Infektion einen nachhaltigen Einfluss auf die Gehirnstruktur von FG hat. Dieser unterschied sich bemerkenswerterweise deutlich vom Einfluss der Frühgeburtlichkeit. Dies weist auf einen eigenständigen und unabhängigen Effekt der beiden Faktoren hin. Das bei den ehemals hCMV-infizierten höhere globale Volumen an grauer Substanz erscheint auf den ersten Blick kontraintuitiv, ist jedoch durch den großflächig dickeren Kortex bei den FG $_{hCMV+}$ erklärbar. Dieser Effekt der Infektion lässt sich wahrscheinlich durch eine Beeinflussung der Hirnreifung erklären, im Sinne einer Interferenz mit der kortikalen Proliferation und/oder Organisation. Dies scheint plausibel angesichts der Neigung von hCMV, bei frühen intrauterinen Infektionen kortikale Aufbaustörungen zu induzieren.

Aus unseren Ergebnissen lässt sich schließen, dass die früh-postnatale hCMV-Infektion bei FG in unserem Kollektiv einen nachhaltigen Einfluss auf die Gehirnstruktur hat. Deshalb sollten Anstrengungen zur Vermeidung einer solchen Infektion weiter forciert werden.

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7 Contribution

This work was conducted at the University Children's Hospital Tübingen under the supervision of Marko Wilke. The study was designed by Marko Wilke with support from Karen Lidzba, Andrea Bevot and Rangmar Goelz. The neurological and neuropsychological assessment was performed by Karen Lidzba and her team, MR data was acquired under the supervision of Marko Wilke and Till-Karsten Hauser.

I was introduced to the methods used in this work by Marko Wilke. All analyses were then conducted by me with the help and supervision of Marko Wilke. For analyses of surface-based data, we considered the advice of Prof. Gaser of Jena.

Following introductions by Marko Wilke, I performed the statistical evaluation of the results, under Marko Wilke's supervision. Advice by Karen Lidzba was considered. The results were discussed and interpreted by Marko Wilke and me.

I assure that this manuscript was written by myself under Marko Wilke's supervision. Figure 1 was based on a publication by Dahnke et al., as marked in the work. Figures 2-15 were created by me under supervision of Marko Wilke. This manuscript was proofread by Marko Wilke. I assure that I only used the references marked in this work.

8 Publication

This work was presented orally by me at the “Neurowoche 2018” and was published online.

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