

From the DEPARTMENT OF WOMEN'S AND CHILDREN'S HEALTH

Karolinska Institutet, Stockholm, Sweden

**BRAIN IMAGING IN EXTREMELY PRETERM INFANTS –
RELATIONS TO PERINATAL FACTORS AND OUTCOME**

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The cover is a visual interpretation of the title and the work performed in this thesis also representing the efforts we as adults must make in order to provide the best possible future outcomes for those entering our world. It was kindly designed for this thesis by my friend Sonia Haritidi, whom I wholeheartedly thank.

“Because children grow up, we think a child's purpose is to grow up. But a child's purpose is to be a child. Nature doesn't disdain what lives only for a day. It pours the whole of itself into the each moment. We don't value the lily less for not being made of flint and built to last. Life's bounty is in its flow, later is too late. Where is the song when it's been sung? The dance when it's been danced? It's only we humans who want to own the future, too. We persuade ourselves that the universe is modestly employed in unfolding our destination. We note the haphazard chaos of history by the day, by the hour, but there is something wrong with the picture. Where is the unity, the meaning, of nature's highest creation? Surely those millions of little streams of accident and willfulness have their correction in the vast underground river, which, without a doubt, is carrying us to the place where we're expected! But there is no such place, that's why it's called utopia. The death of a child has no more meaning than the death of armies, of nations. Was the child happy while he lived? That is a proper question, the only question. If we can't arrange our own happiness, it's a conceit beyond vulgarity to arrange the happiness of those who come after us.”

— Tom Stoppard, *The Coast of Utopia*

“Without deviation from the norm, progress is not possible.”

— Frank Zappa

Dedicated to my beloved parents who have always been by my side

ABSTRACT

The survival of extremely preterm infants has improved over the last decades along with the advances in perinatal care. These immature children are born at a vulnerable stage of brain development, before entering the third trimester of gestation, and are at an increased risk for brain injuries, atypical brain development and subsequent adverse neurodevelopment.

The overall aim of the works compiled in this thesis was to qualitatively and quantitatively study brain structure and volumes in extremely preterm infants at term equivalent age, and to investigate associations with neonatal risk factors and with infant and toddler age outcomes.

Infants born before 27 gestational weeks in Stockholm during a four-year period were eligible for these studies. They underwent conventional Magnetic Resonance Imaging and Diffusion Tensor Imaging at term equivalent age. Conventional T1- and T2-weighted images were visually inspected using a scoring system for white and grey matter abnormalities. Automatic segmentation and Voxel-based morphometry with DARTEL registration were used to analyze global and regional volumes of grey and white matter. White matter microstructure was investigated with Diffusion Tensor Imaging, and analyzed using Tract-Based Spatial Statistics and Region of Interest analysis.

In Paper I we investigated whether prematurity per se or perinatal risk factors could explain altered brain structure after preterm birth in extremely preterm infants without focal brain lesions on visual inspection of MRI. Brain white matter microstructural differences were investigated between extremely preterm infants and term born healthy controls using Tract-Based Spatial Statistics and subsequently associations with perinatal risk factors were explored. White matter microstructure was influenced by preterm birth and by neonatal respiratory factors, whereas the degree of prematurity within the extremely preterm range (23 – 26+6 weeks) appeared to be of less importance within the narrow range.

In Paper II, the incidence of another possible, frequently present but inadequately studied, perinatal risk factor for adverse brain development, hyperglycemia, and its relation to mortality and white matter abnormalities on visual inspection of conventional MRI was studied. Hyperglycemia on the first day after birth was identified as an independent risk factor for increased mortality rates and brain damage, in terms of white matter reduction.

In Paper III, the relationship between white matter microstructural and morphometric brain differences at term equivalent age and hyperglycemia in extremely preterm infants was explored with Tract-Based Spatial Statistics and Voxel-based morphometry. Early hyperglycemic exposure was associated with altered diffusion measures in major white matter tracts and reduction of regional white and grey matter volumes.

In Paper IV, sex differences in brain development and neurodevelopmental outcome in children born extremely preterm were studied. In addition, associations with neonatal brain morphology were assessed with conventional structural and diffusion MRI. Sex

related differences were observed on neonatal structural MRI, including differences in the patterns of correlations between brain volumes and developmental scores at both global and regional levels. Cognitive and language outcome at age 30 months was poorer in boys than in girls.

LIST OF PUBLICATIONS

- I. **Georgios Alexandrou**, Gustaf Mårtensson, Beatrice Skiöld, Mats Blennow, Ulrika Ådén, Brigitte Vollmer. White Matter Microstructure Is Influenced By Extremely Preterm Birth And Neonatal Respiratory Factors. *Acta Paediatr.* 2013 Oct 4. doi: 10.1111/apa.12445.
- II. **Georgios Alexandrou**, Beatrice Skiöld, Jonna Karlén, Mesfin K. Tessma, Mikael Norman, Ulrika Ådén and Mireille Vanpée. Early Hyperglycemia Is A Risk Factor For Death And White Matter Reduction In Preterm Infants. *Pediatrics* 2010;125:e584; doi: 10.1542/peds.2009-0449.
- III. **Georgios Alexandrou**, Nelly Padilla, Gustaf Mårtensson, Finn Lennartsson, Mirelle Vanpee, Brigitte Vollmer, Ulrika Ådén. White Matter Microstructure And Brain Growth In Extremely Preterm Infants With Early Hyperglycemia. *Manuscript*
- IV. Béatrice Skiöld, **Georgios Alexandrou**, Nelly Padilla, Mats Blennow, Brigitte Vollmer, Ulrika Ådén. Sex Differences In Outcome And Associations With Neonatal Brain Morphology In Extremely Preterm Children. *Submitted manuscript*

TABLE OF CONTENTS

1	BACKGROUND.....	1
1.1	Preterm Birth	1
1.1.1	Survival.....	1
1.2	Neonatal Morbidities	2
1.3	Perinatal Brain Development	3
1.3.1	Typical brain development.....	3
1.3.2	Oligodendrocyte development and myelination.....	5
1.3.3	Structural brain development in preterm birth.....	6
1.3.4	Respiratory disease in EPT and brain development	8
1.3.5	Glucose	10
1.3.6	Sex	13
1.4	Investigation of the preterm brain with MRI	14
1.4.1	Magnetic resonance imaging	14
1.4.2	Diffusion MRI	15
1.4.3	Brain Tissue Segmentation, Volumetry and Voxel-based morphometry	17
1.4.4	Voxel-based morphometry-DARTEL	18
1.5	Neurological and developmental outcome following preterm birth.....	18
1.5.1	Neurological and neuromotor outcomes.....	18
1.5.2	Neurodevelopmental, cognitive and behavioral outcomes.....	19
1.5.3	MRI at TEA and relation to neurodevelopment	20
2	AIMS	21
3	PARTICIPANTS AND METHODS	22
3.1	Study Design.....	22
3.2	Ethical Considerations	22
3.3	Participants	22
3.3.1	Preterm Infants	22
3.3.2	Term born control infants.....	23
3.4	Methods, an overview of subjects, methods and outcome	24
3.4.1	Magnetic Resonance Imaging.....	24
3.4.2	Diffusion MRI	26
3.4.3	Neurodevelopmental follow up.....	27
3.4.4	Glucose monitoring, documentation and scoring systems	27
3.5	Statistical Analysis	28
4	RESULTS AND DISCUSSION.....	31
4.1	Survival And Neonatal Morbidities	31
4.2	Brain imaging findings in extremely preterm infants at term equivalent age	31
4.2.1	Findings on visual inspection of conventional structural MRI (Papers I, IV)	31
4.2.2	Diffusion MRI findings (Paper I, III, IV)	32
4.2.3	Volumetric MRI findings (Paper III, IV).....	34

4.3	Risk Factors and outcomes	36
4.3.1	Hyperglycemia and Mortality (Paper II)	37
4.3.2	Hyperglycemia, sex and Morbidities.....	37
4.4	Brain Abnormalities (Paper I, II, III, IV).....	38
4.4.1	Hyper- and hypoglycemia and Brain Abnormalities	38
4.4.2	Respiratory Illness, Immaturity, Sex and Brain abnormalities	40
4.4.3	Sex and Neurodevelopmental outcome (Paper IV).....	41
5	CONCLUSIONS	44
6	GENERAL DISCUSSION	45
7	ACKNOWLEDGEMENTS	47
8	REFERENCES	49

LIST OF ABBREVIATIONS

ADC	apparent diffusion coefficient
AD	axial diffusivity
BPD	bronchopulmonary dysplasia
BSID-III	Bayley Scales of Infant and Toddler Development Third Edition
BW	birth weight
CC	corpus callosum
CI	confidence interval
CRIB	Critical Risk Index for Babies
CR	corona radiata
CSO	centrum semiovale
DTI	diffusion tensor imaging
EC	external capsule
EPT	extremely preterm
FA	fractional anisotropy
GA	gestational age
GM	grey matter
ILF	inferior longitudinal fasciculus
IVH	intraventricular hemorrhage
MD	mean diffusivity
MRI	magnetic resonance imaging
PDA	patent ductus arteriosus
RD	radial diffusivity
ROI	region-of-interest
SD	standard deviation
TEA	term equivalent age
TBSS	Tract-Based Spatial Statistics
WM	white matter
VBM	Voxel-based morphometry

1 BACKGROUND

1.1 PRETERM BIRTH

Preterm birth is defined as birth prior to 37 weeks gestational age (GA). Extremely preterm (EPT) birth is defined as birth prior to 28 weeks GA. i.e. before the beginning of the third trimester. The present study is based on the national Swedish EXPRESS study, which includes infants born before 27 weeks GA, accounting for 2.3 per 1000 live births per year in Sweden (Fellman V et al. 2009).

In half of preterm births the cause for preterm birth is never determined, making the reduction of preterm birth a challenge. The main categories of causes of preterm birth are preterm labor induction, and spontaneous preterm labor accompanied by premature rupture of the fetal membranes. A number of etiologies have been associated with preterm birth including precocious fetal endocrine activation, uterine overdistension (placental abruption), decidual bleeding, and intrauterine inflammation/infection (Simhan HN and SN Caritis 2007). Infections play a major role in the genesis of preterm birth and may account for 25–40% of events (Goldenberg RL et al. 2000). The frequency of infection in preterm birth is inversely related to gestational age (Goldenberg RL et al. 2008). Intrauterine growth retardation, complications of multiple pregnancies, and maternal conditions such as high blood pressure (Goldenberg RL et al. 1998), pre-eclampsia (Banhidy F et al. 2007), maternal diabetes (Rosenberg TJ et al. 2005), asthma, thyroid disease, and maternal heart disease also increase the risk of preterm birth.

1.1.1 Survival

Preterm neonates are at elevated risk of neonatal mortality. It is encouraging, however, that survival rates following EPT birth have greatly improved worldwide during the last decades (Saigal S and LW Doyle 2008). Short-time survival rates have increased with the application of better ultrasound diagnostics for assessment of fetal well-being, the use of antenatal corticosteroids and exogenous surfactant, delay of delivery with tocolytics, and possibly the use of cesarean section at early gestational ages on fetal indication, advanced ventilator strategies as well as the centralization of neonatal care units (NICU).

A study of extremely low birth weight infants (birth weight less than 1000 grams) in Australia presented a threefold increase in survival rates, from 25% in 1979 to 73% in 1997 (Doyle LW 2004). Survival rates of infants with a GA at birth less than 26 weeks in the United Kingdom and Ireland were shown to increase from 39% in 1995 to 47% in 2006 (Costeloe K et al. 2000; Costeloe K 2006). The EXPRESS study in Sweden showed a 1-year survival of 70% of live born infants below 27 weeks. Mortality rates in the EXPRESS study were inversely related to GA at birth, ranging from 93% at 22 weeks GA to 24% at 26 weeks GA (Fellman V et al. 2009). There has been a 20-30% increase in survival rates of EPT infants born in Sweden with gestational ages above 23 weeks (Hakansson S et al. 2004; Fellman V et al. 2009).

Differences in mortality rates between countries and regions can partly be accounted for by different practice styles regarding delivery, resuscitation, initial stabilization at birth and during the first hours after admission to the NICU (Field D et al. 2009). Factors such as parental educational and socio-economical status, maternal background, patient population, or genetic factors may have an impact as well.

In systematic prediction models, the combination of different factors that contribute to neonatal mortality and morbidity includes being of average size for GA, sex, ethnicity, absence/presence of serious congenital malformations, use of antenatal steroids, temperature on admission, and respiratory status (Medlock S et al. 2011).

The work presented in this thesis focused on three previously recognized risk factors for mortality, brain injury, atypical brain development and neurodevelopmental morbidity, namely respiratory factors, sex, and immaturity at birth. In addition, we have studied the associations between hyperglycemia, which is a potential risk factor, and mortality and brain injury.

1.2 NEONATAL MORBIDITIES

EPT infants commonly experience acute lung disease (respiratory distress syndrome) and chronic lung disease (CLD; bronchopulmonary dysplasia, BPD). Gastrointestinal problems include feeding difficulties and necrotizing enterocolitis (NEC). Impaired circulation secondary to patent ductus arteriosus (PDA) may arise. Hematologic problems include anemia of prematurity, thrombocytopenia and hyperbilirubinemia. Retinopathy of prematurity (ROP) is frequently seen as well. Other perinatal and neonatal factors that affect outcome are postnatal corticosteroid use, postnatal infection (Bassler D et al. 2009), multiple births (Laptook AR et al. 2005) and no tocolysis (EXPRESS 2010). There is a high risk for brain hemorrhage (germinal matrix hemorrhage, intraventricular hemorrhage (IVH) and parenchymal hemorrhagic infarction (PHI), which may be followed by posthemorrhagic hydrocephalus and non-cystic white matter disease (Volpe JJ 2008). The incidence of focal periventricular white matter lesions (periventricular leukomalacia, PVL) has decreased over the past decade (Rutherford MA et al. 2010). However, non-focal, widespread white matter abnormalities have been observed in a high proportion of preterms, in particular, in the most immature infants (Volpe JJ 2009).

1.3 PERINATAL BRAIN DEVELOPMENT

The normal formation of our neural circuitry is an ongoing process that continues after birth. During gestation it is the result of numerous well-orchestrated spatial and temporal steps - neurogenesis, neuronal migration, synaptogenesis etc. Figure 1 is a schematic illustration of the timing of the major neurodevelopmental processes.

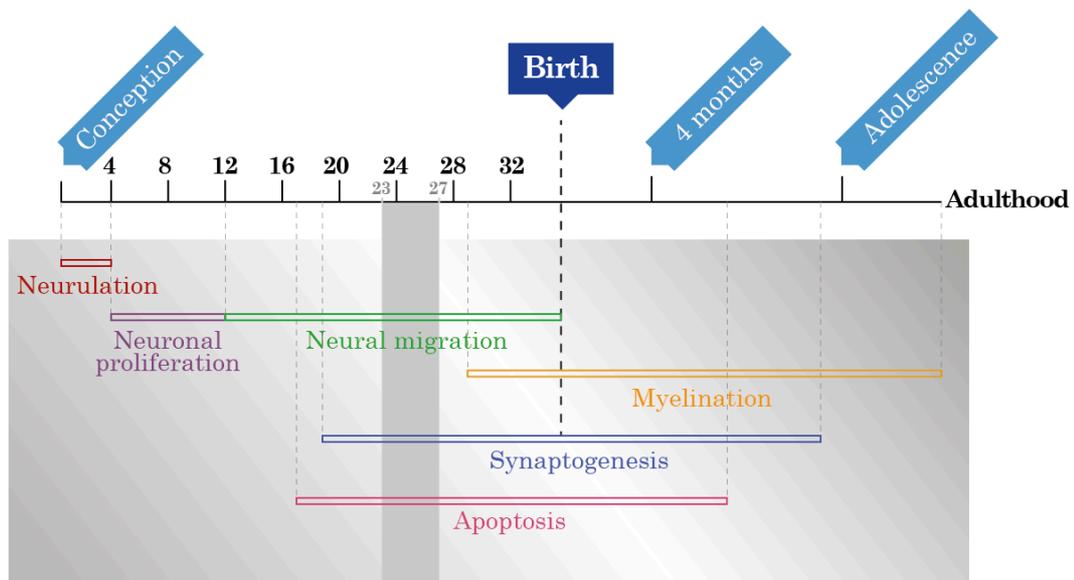


Figure 1. Schematic representation of the activity of neurodevelopmental processes in the human brain modified from Tau GZ and BS Peterson 2010).

1.3.1 Typical brain development

The perinatal and early postnatal period is characterized by rapid brain development (Figure 2). Macroscopically, from the end of the second trimester until birth, the cerebral cortex goes from a lissencephalic state having only a rudimentary central and parieto-occipital cortex to the highly convoluted sulcation pattern of the adult brain. The subplate zone, a transient fetal brain structure serving as a waiting compartment for growing cortical afferents migrating to the cortical plate (Volpe JJ 2008), is initially visible on magnetic resonance imaging (MRI) scans performed early in gestation, but absent as term age approaches (Rados M et al. 2006). Similarly, the radial organization of the cortical plate is initially evident on MRI, but changes to a more heterogeneous structure as the cortex develops (McKinstry RC et al. 2002).

Postmortem and *in-vivo* fetal imaging studies in humans in combination with histochemical imaging studies in animals show that early brain development follows an organized pattern (Yacovlev P LA 1967; Brody BA et al. 1987; Bockhorst KH et al. 2008; Vasung L et al. 2010; Rajagopalan V et al. 2012). The germinal matrix in the periventricular zone has been identified as early as 12 weeks of gestation in postmortem anatomical studies of human brain development (Meng H et al. 2012). The lower corticospinal tract and limbic fibers in the fornix and stria terminalis are centrally located WM tracts and have been identified in a diffusion MRI postmortem study of anatomical brain development at 13 weeks of gestation (Huang H et al.

2009). During the second trimester, commissural, projection, and some association tracts were identified; the corpus callosum and internal capsule have been identified at approximately 15 weeks of gestation. Association fibers of the sagittal striatum and the external capsule are seen at 13–15 weeks. The cerebral peduncle and internal capsule developed earlier than the more peripheral regions that extended into the corona radiata at 19–20 weeks (Huang H et al. 2009). Cortical thickening and deepening of sulci have been shown to continue during the second and third trimesters (Zhang Z et al. 2011; Zhang ZH et al. 2013).

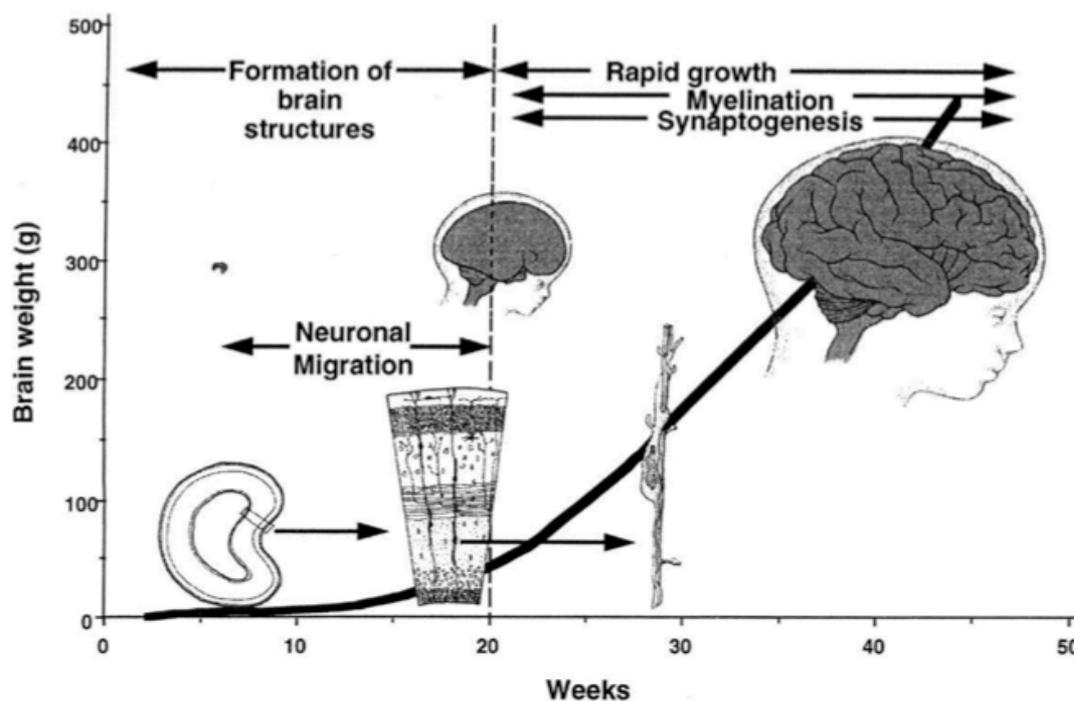


Figure 2. Schematic representation of processes during brain development; from conception to birth, Courtesy of M. Squiers, modified.

The first main phase of cellular proliferation includes mainly neurons and occurs from 8 to 16 weeks of gestation; from around 20 weeks the second phase occurs and this is characterized by glial cell proliferation. Initial cell production is in the ventricular zone (germinal matrix), which initially contains "progenitor" cells that subsequently divide to produce the postmitotic neurons and glia. Gliogenesis and neurogenesis are then thought to take place in the subventricular zone (Volpe JJ 2001). Neural progenitor proliferation regulation is through the principal inhibitory (gamma aminobutyric acid, GABA) and excitatory (glutamate) neurotransmitters (Haydar TF et al. 2000).

Then radial glial cells form a link between the ventricular zone and the pial surface, where neurons migrate towards the margins of the cerebral hemispheres to form the cortex. The preplate, which is situated at the outer margin of the cerebral hemispheres, is where the earliest formed cells accumulate. The preplate is subsequently divided into the marginal zone, at the pial surface, and the subplate. This allows migration of additional, newly formed neurons form the cortical plate, between the marginal zone and the subplate. Formation of the cortex begins from the inner layer prior to the outer layers and migrating cells pass through earlier formed layers to the margin of the

cortical plate to ultimately form six histologically distinct layers parallel to the cortical surface (Volpe JJ 2008).

The subplate zone is a layer of the cerebral wall that develops at around 13 weeks and gradually disappears after 32-34 gestational weeks. Neurons that form the subplate zone migrate to the marginal zone before true cortical neurons migrate to the cortical plate and are critical to cortical organization. They form part of the pre-plate zone before it splits. Subplate neurons rapidly differentiate and develop a dendritic tree with spines expressing receptors for various chemical mediators. This enables them to form reciprocal connections to the thalamus and cerebrocortex. Thus, subplate neurones form a functional synaptic link for 'waiting' thalamo-cortical and cortico-cortical afferents, whose neuronal targets have not yet arrived at the cortical plate (Kostovic I and N Jovanov-Milosevic 2008).

Axons group together to form bundles and these develop into projection fibers (uniting the cortex with the lower parts of the brain and with the spinal cord), association fibers (uniting different parts of the same cerebral hemisphere) and commissural fibers (connecting corresponding regions between the two hemispheres of the brain) (Clarke S et al. 1989), many of which are subsequently pruned. Astroglial cells and macrophages are first detected between 25 and 44 weeks gestation, prior to the full myelination of these structures (Carpenter MB and J Sutin 1983).

1.3.2 Oligodendrocyte development and myelination

Oligodendrocytes are a class of glial cells that myelinate axons. Their role is crucial in the efficient transmission of electrical signals along the axon. In the white matter of the central nervous system they are the predominant glial cell type.

The cells arise from the precursor oligodendrocyte cells generated in the proliferative ventricular and subventricular zones during the last months of gestation and the early postnatal period (Niehaus A et al. 1999; Back SA et al. 2007). As these cells migrate away from these periventricular germinal regions and into the white matter, they pass a series of maturational stages until they finally differentiate into mature oligodendrocytes capable of myelination (Figure 3).

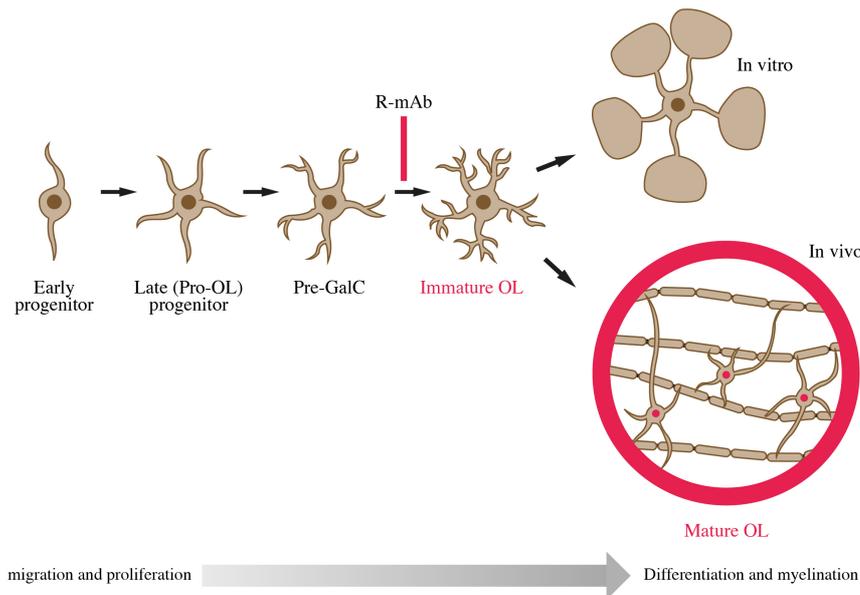


Figure 3. Schematic representation of oligodendrocyte development and myelin biogenesis, modified from N. Jackman 2009.

Myelination allows the transmission of neural impulses through the nervous system. It begins around the start of the second trimester of pregnancy (Gillespie MJ and RB Stein 1983) and the entire process is not complete until adulthood at about 25–30 years of age (Fields RD 2008). The process initiates with the proliferation of a population of glial cells, which differentiate into oligodendrocytes and align along neuronal axonal projections. Myelination begins in the primary motor and sensory areas (the brain stem and cortex) and gradually progresses to the areas that subservise higher cognitive functions. During the first year, myelin spreads through the entire brain and follows a specific spatio-temporal pattern. Myelination typically proceeds in a central to peripheral, inferior to superior and posterior to anterior manner (Barkovich AJ 2005).

1.3.3 Structural brain development in preterm birth

1.3.3.1 Brain structure at term equivalent age (TEA)

Preterm birth poses a risk for brain injury and for atypical brain development (Volpe JJ 2009).

Neuroimaging, and in particular MRI has been used to delineate patterns of brain injury after preterm birth at term equivalent age (TEA), in infancy, in childhood and adolescence. Findings on visual inspection of conventional structural T1-weighted and T2-weighted images include abnormal signal intensity involving the periventricular white matter, periventricular cysts with or without focal or punctate hemorrhages, ventricular dilatation, and periventricular white matter reduction, and thinning of the corpus callosum.

At TEA, a neuroimaging study has demonstrated a unique pattern of cerebral abnormality consisting of global white matter atrophy, ventriculomegaly, immature gyral development, and enlarged subarachnoid spaces, in the majority of the sample in those born <26 weeks of gestation (Inder TE et al. 2003). Delayed cortical

development and decreased cortical volumes have been reported (for review see Keunen *et al* (Keunen K *et al.* 2012)). It has been proposed that diffuse excessive high-signal intensity (DEHSI) at TEA in the periventricular white matter on T2-weighted images represents the diffuse component of white matter injury in preterm infants. DEHSI is a common finding in preterm infants (Skiold B *et al.* 2010) and has also been associated with a decrease in central GM volume (Boardman JP *et al.* 2006; Srinivasan L *et al.* 2007). In the population based cohort in Stockholm on which the work of this thesis is based, it was previously shown that no or mild white abnormalities were present in 86% of infants, and 14% had moderate or severe WM abnormalities. DEHSI, present in 56 % of the infants, were seen in infants with all grades of white matter abnormalities (Skiold B *et al.* 2010).

Quantitative MRI studies at TEA using diffusion weighted MRI and Diffusion Tensor Imaging (DTI) have shown that preterm birth is associated with widespread alterations in white matter microstructure, including the major projections and association fibres (Anjari M *et al.* 2007; Rose SE *et al.* 2008). Some of these studies suggest that prematurity as such, independent of risk factors, results in atypical white matter development (Huppi PS *et al.* 1998; Dudink J *et al.* 2007; Hasegawa T *et al.* 2011). Such changes in white matter structure appear to be persisting as indicated by a number of studies in childhood and adolescence (Vangberg TR *et al.* 2006; Skranes J *et al.* 2007; Constable RT *et al.* 2008; Nagy Z *et al.* 2009; Mullen KM *et al.* 2011; Groeschel S *et al.* 2013).

Volumetric studies, either assessing total brain tissue volumes, or using high resolution 3D images for automated analysis of regional brain volumes, have shown that at TEA global as well as regional volumes are different in preterm infants when compared to term born infants (Peterson BS *et al.* 2003; Inder TE *et al.* 2005; Limperopoulos C *et al.* 2005; Mewes AU *et al.* 2006; Shah DK *et al.* 2006; Srinivasan L *et al.* 2006; Zacharia A *et al.* 2006; Mewes AU *et al.* 2007; Srinivasan L *et al.* 2007; Thompson DK *et al.* 2007; Thompson DK *et al.* 2008; Thompson DK *et al.* 2009). Differences have been shown in cortical grey matter (GM), subcortical GM, the myelinated white matter (WM), and the cerebellum along with an increase in cerebrospinal fluid. It is noteworthy, that a correlation has been shown between the degree of immaturity at birth and reduction in brain tissue volumes (Inder TE *et al.* 2005; Limperopoulos C *et al.* 2005; Boardman JP *et al.* 2006; Srinivasan L *et al.* 2006; Zacharia A *et al.* 2006; Boardman JP *et al.* 2007). It has been suggested that GA at birth remains independently associated with smaller cerebral volumes when correcting for other confounding factors such as the presence of cerebral WM injury (Inder TE *et al.* 2005) or when correcting for both intracranial and total brain volumes (Limperopoulos C *et al.* 2005). This is supportive of the notion of an inherent adverse effect of prematurity on brain development (Inder T *et al.* 2005; Limperopoulos C *et al.* 2005).

More recently, cerebellar abnormalities have been described in a number of studies. However, there are somewhat inconsistent findings in the literature. One study reported no evidence for a primary reduction in cerebellar development in relation to prematurity, although there was evidence for a secondary effect of cerebral white matter injury on cerebellar development independent of immaturity (Shah DK *et al.* 2006) This is in agreement with a study reporting associations of reduced cerebellar volumes with pathology such as hemorrhagic parenchymal infarction, intraventricular hemorrhage (IVH) with dilation, and periventricular leukomalacia (PVL) (Srinivasan L *et al.* 2006).

There has also been a suggestion that cerebral volume is not reduced during intensive care for the majority of preterm infants, however prolonged need of supplemental oxygen is a risk factor for reduction of global brain growth (Boardman JP et al. 2007).

Regarding cortical GM volumes as well as WM volumes, studies have described both region-specific increases and decreases (Peterson BS et al. 2003; Thompson DK et al. 2007). Peterson *et al.* presented enlarged volumes in the anterior parts of the cortical GM (Peterson BS et al. 2003; Thompson DK et al. 2007), supporting the results from their prior study (Peterson BS et al. 2000) in 8-years-old preterm born children where they showed significantly larger prefrontal cortical GM regions in children born prematurely compared to term controls. Increase in cerebrospinal fluid (CSF) has been a consistent finding in nearly all cerebral regions in several studies (Mewes AU et al. 2006; Mewes AU et al. 2007; Thompson DK et al. 2007).

A number of imaging studies in childhood and adolescence have shown differences in both GM and WM volumes and density (for review see Kieviet *et al.* (de Kieviet JF et al. 2012). Alterations in both WM and GM volumes have been shown to be associated with impairments in a variety of motor, cognitive and behavioral functions (Nosarti C et al. 2008; Lowe J et al. 2011).

1.3.4 Respiratory disease in EPT and brain development

The lungs are one of the main organs greatly affected by premature birth, as they are one of the last organs to mature in the womb. The majority of morbidity and mortality in infants born preterm result from respiratory complications, secondary to biochemical and structural immaturity of their lungs, and their insufficient respiratory drive.

Acute lung disease requiring mechanical ventilation and surfactant therapy is frequent in this population. It has an incidence of about 50% in infants born before 30 weeks of gestation (Ramanathan R and S Sardesai 2008). The normal process of alveolar and vascular development is disrupted. Histological lung abnormalities, of premature infants born during the canalicular and saccular stages of lung development, comprise reduced functional surface area, insufficient alveolarisation and fibrin deposition in the air spaces (Sinha SK et al. 2008). The principal physiological abnormality of acute lung disease is reduced surfactant production. This leads to an increased alveolar surface tension and subsequent collapse, atelectasis and decreased lung compliance. Antenatal steroid therapy and postnatal surfactant administration are the treatments of choice.

Ventilation and oxygen rich gas therapies are life-saving treatments and have been shown to reduce morbidity and mortality in infants born preterm (Birenbaum E et al. 1983), but also to promote lung injury. This may lead to long-term pulmonary insufficiency, and 30-40% of preterm infants develop chronic lung disease. A combination of the preterm infant's abnormal respiratory function and the iatrogenic consequences of treatment can lead to periods when O₂ and CO₂ levels are surpassing or beneath the normal range. This may precipitate retinopathy of prematurity (Kim TI et al. 2004) and interfere with normal growth and development (Sinha SK et al. 2008).

Mechanical ventilation has been associated with adverse brain development (Anjari M et al. 2009; Ball G et al. 2010; Bonifacio SL et al. 2010). Increasing duration of mechanical ventilation was recently shown to be associated with delayed maturation of the occipital periventricular zone and centrum semiovale (Pogribna U et al. 2013).

Chronic lung disease, also known as bronchopulmonary dysplasia (BPD), is typically described as requirement of supplementary oxygen at 36 weeks' gestational age. Affected infants may require supplemental oxygen for months, and although few remain oxygen dependent beyond two years of age (Greenough A et al. 2002; Greenough A 2006), respiratory symptoms, reflecting disturbed lung growth, are often evident for many years (Greenough A 2006). BPD commonly arises when the immature developing lungs of preterm infants are subjected to recurring injury, which is thought to result from hypo- or hyper-inflation of the developing alveoli, leading to inflammation and disruption of normal development. Transient accumulation of fluid in the lung or permanent reductions in alveolarisation may follow. Frequently, this injury arises from, or is exacerbated by, mechanical ventilation. More than 30% of preterm babies born prior to 30 weeks gestational age develop neonatal BPD characterized by alveolar simplification, dysmorphic capillaries, and increased in vascular and airway smooth muscle cells (Margraf LR et al. 1991; Husain AN et al. 1998; Jobe AH and E Bancalari 2001; Thebaud B and SH Abman 2007). Infants with BPD are at increased risk for long-term hospitalization and recurrent respiratory complications in early infancy, as well as life-long consequences from impaired pulmonary development (Jobe AH and E Bancalari 2001; Greenough A et al. 2002; Doyle LW et al. 2005; Ehrenkranz RA et al. 2005; Baraldi E and M Filippone 2007).

The cause of BPD is multifactorial, nevertheless the pre- and postnatal factors responsible for the disrupted alveolar growth are well known. BPD is strongly associated with preterm birth; prenatal infection and inflammation, mechanical ventilation, oxygen toxicity with decreased host antioxidant defenses, patent ductus arteriosus and postnatal infection all contribute to the pathogenesis of BPD. Recently, preeclampsia on its own has been defined as a risk factor for the development of BPD (Hansen AR et al. 2010).

Respiratory disease is associated with adverse neurological outcome (Anderson PJ and LW Doyle 2004; Doyle LW et al. 2005). Preterm infants with supplemental oxygen needs at 28 days postnatal life show reductions in cerebral volume at TEA (Boardman JP et al. 2007); preterms requiring oxygen at 36 weeks' corrected age have been shown to have reduced growth throughout the brain (Thompson DK et al. 2007). It has also been shown that white matter microstructure as assessed with diffusion tensor imaging is altered in BPD compared with those with no need for supplemental oxygen (Anjari M et al. 2009) and that these alterations occur in several white matter tracts (Ball G et al. 2010).

The use of antenatal steroids to accelerate lung maturation, the development of surfactant replacement therapy for acute respiratory failure, the institution of lung protective strategies of ventilation, and an optimization of nutritional support have all contributed to an overall decrease in the mortality and reduction in brain injuries in EPT infants (Foix-L'Helias L et al. 2008).

1.3.5 Glucose

1.3.5.1 *Endocrine Response at Birth*

Prenatally, the fetuses' main energy source is glucose. It is provided via constant transplacental infusion by insulin-independent glucose transporters (Ogilvy-Stuart AL and K Beardsall 2010). In term pregnancies during the last trimester there is an increase in maternal glucose production. At this point 40% of the fetuses' glucose is converted to glycogen in the liver and muscle or to lipids (Ogilvy-Stuart AL and K Beardsall 2010). During the last trimester, the fetus prepares for a time after the end of continuous glucose supply mainly via an increase of glycogen synthesis and storage. Storage of glycogen is a process, which starts approximately at 27 weeks GA, due to corticoid effects in the third trimester. Thus, the EPT infant is at a disadvantage, with regard to hepatic glycogen stores being limited at the time of birth.

1.3.5.2 *Perinatal glucose metabolism in term born infants*

The liver, hormones and other mechanisms control glucose metabolism after birth. 'Physiological' stress after birth is expressed by high plasma catecholamine levels, mobilization of glycogen, and an increase in glycogen receptors. These result in low blood glucose levels (Hey E 2005; Mitanchez D 2007). Glucagon is secreted from the pancreas with the decrease of insulin concentrations. Glucagon and catecholamines have synergetic effects with glucocorticoids. In parallel, glycogenolysis, lipolysis and gluconeogenesis occur and gluconeogenic substrates are delivered through muscle protein breakdown (Hey E 2005; Mitanchez D 2007). During the first hours after birth the only source of glucose is the hepatic output by glycolysis and gluconeogenesis. High glucagon and low insulin plasma levels activate gluconeogenesis, which begins in the second hour after birth and peaks at 12 hours after birth contributing to the energy supply of brain and vital organs (Hey E 2005; Dumortier O et al. 2007; Mitanchez D 2007). Energy requirements are high and glucose can only provide 70% of the cerebral energy transformation thus alternative substrates are utilized (Hawdon JM et al. 1992; Hawdon JM et al. 1995). Lactate and ketone bodies are alternative fuels to glucose. Ketogenesis rises significantly in the first 24 hours after birth resulting in high ketone concentrations (Hawdon JM et al. 1992; Hawdon JM et al. 1995).

1.3.5.3 *Perinatal glucose metabolism and energy metabolism in preterm infants*

Glycogen is the preterm infant's major source of glucose. Since the storage of glycogen only occurs in the third trimester, EPT infants have a very limited hepatic glycogen store at birth (Hey E 2005; Mitanchez D 2007). Induction of gluconeogenesis requires time and is dependent on mature enzymes, which like glucose-6-phosphate are low in preterm infants (Hawdon JM et al. 1992; Hawdon JM et al. 1995). Glycogen is mobilized through glycogenolysis, which is activated by increased levels of glucagon. Glucagon has been intravenously given to the preterm infant and reported as a safe and effective alternative to high doses of glucose in case of persistent hypoglycemia during the first 7 days after birth. Fat stores are extremely low in preterm infants; only 2% of their body weight and supplementary energy supply through ketogenesis is limited. Despite ketogenesis being triggered through feeding (Hays SP et al. 2006), the enteral alimentation is limited due to minor volumes and gastrointestinal immaturity. Enzyme inactivity also reduces the sufficiency of ketogenesis and lipolysis to supply enough energy (Mitanchez D 2007). Lipids cause an increase in glucose utilization. In the very low birth weight infant born extremely preterm increased gluconeogenesis was shown (Sunehag AL 2003) through parenteral lipid emulsion via hydrolysis of glycerol with sequential conversion into glucose.

Parenteral energy supply with continuous glucose infusion is required to prevent hypoglycemia and maintain normoglycemia. This, however, could risk causing hyperglycemia, commonly observed in the preterm infant during the first week after birth.

1.3.5.4 Hypo- and Hyperglycemia

Hypoglycemia, frequently seen in the preterm infant, may cause cerebral energy failure, impaired cardiac performance, muscle weakness, glycogen depletion, and diminished glucose production. Lower plasma glucose levels found in the preterm infant have been suggested to reflect the current nutritional management of these small infants (Cornblath M and R Schwartz 1976). Many different definitions of hypoglycemia may be found in the literature varying from < 2 mmol/L blood (< 36 mg/dL) to < 2.6 mmol/L (< 50 mg/dL) plasma (Aynsley-Green A and JM Hawdon 1997; Cornblath M and R Ichord 2000; Kalhan S and S Peter-Wohl 2000).

Hyperglycemia is also recognized as a common condition in preterm infants, and is attributable to altered metabolism associated with immaturity, as well as the need for continuous parenteral nutrition (Hey E 2005). In EPT infants receiving IV glucose infusions, hyperglycemia is probably secondary to an insufficient processing of pro-insulin by the immature pancreas and decreased insulin sensitivity of the liver. Therefore, the infant continues their glucose production despite hyperglycemia (Mitanchez-Mokhtari D et al. 2004). Hyperglycemia is frequently found in the first week after birth, with an increased frequency being associated to lower GA and birth weight, as well as to severe clinical situations. It can be triggered by respiratory distress (Lilien LD et al. 1979), surgery (Anand KJ et al. 1985), neonatal pain, sepsis, and other stressful events (Louik C et al. 1985). Despite its frequency in this population it is still unknown which plasma glucose level may lead to subsequent neurologic damage.

Definitions of hyperglycemia can be categorized according to a clinical and/or statistical approach. From a functional and clinical perspective, hyperglycemia is defined as a physiological response, which aims to maintain a cerebral metabolism in stress situations. In such a case, treatment with insulin could be used in the presence of glycosuria with osmotic diuresis and dehydration (Hey E 2005). This definition is not useful in EPT infants, who have a varying threshold for glycosuria. A literature review shows different statistical definitions of hyperglycemia, that is, plasma glucose levels of > 7.6 mmol/L (Young B et al. 1989; Hey E 2005) > 8.3 mmol/L (Hays SP et al. 2006; Kao LS et al. 2006) or > 10 mmol/L (Blanco CL et al. 2006; Kao LS et al. 2006). Therefore we tested these three cutoff levels for sensitivity and specificity, with death as the outcome, in paper II and subsequently used 8.3 mmol/L as the cut off.

1.3.5.5 Etiology

Recent studies have added to our understanding of the causes of hypoglycemia due to hyperinsulinism. The identification of hyperinsulinism is essential as management may be more aggressive because insulin inhibits the mobilization of alternative fuels for cerebral metabolism (Rozance PJ and WW Hay 2006). The most frequent cause of hyperglycemia, in the EPT infant population, is the excessive administration of IV glucose. Iatrogenic cases from an inadvertent bolus from flushing an IV line as well as factitious hyperglycemia occurring when blood was drawn from an IV line containing glucose must be ruled out. Pharmaceutically induced hyperglycemia may follow a high-dose of postnatal steroids, vasoactive drugs and theophylline (Hey E

2005).

1.3.5.6 Incidence

The aforementioned lack of consensus in the statistical definition of hyperglycemia has led to a wide range of reported incidences in the literature, which vary from 20 to 80% (Ng SM et al. 2005). In the NIRTURE study, analysis of the control group (not receiving prophylactic insulin) during the first week after birth, hyperglycemia (defined as glucose levels > 145 mg/dL (8 mmol/L) during more than 10% of the time) developed in 80% of very low birth weight infants (Beardsall K et al. 2010). Furthermore, 32% had glucose levels > 180 mg%. (10 mmol/l). This is in accordance with findings from our studies where hyperglycemia (defined as plasma glucose > 8.3mmol/L) was seen in 81% of the EPTs during the first week after birth (Alexandrou G et al. 2010).

1.3.5.7 Pathophysiological basis

The intervening mechanisms for hyperglycemia in preterm infants are very complex, with several inter-related factors such as: hepatic and pancreatic immaturity, insulin resistance, external glucose supply, increased catecholamines, stress, drug use (inotropic drugs, xanthine, corticosteroids) and lack of enteral supply (leads to insulin secretion).

Insulin increases the uptake and utilization of glucose, while inhibiting gluconeogenesis. As described by Hey *et al.* “Insulin facilitates amino acid entry into muscle and protein synthesis, enhances fat synthesis in the liver and glucose uptake by adipose tissue and influences growth and lipogenic activity (as is exemplified by the appearance of babies born to mothers with poorly regulated diabetes at birth)” (Hey E 2005). The preterm neonate responds to hyperglycaemia by secreting proinsulin peptides, which are identified as ‘insulin’ by standard assays but they are of variable biological potency (Hawdon JM et al. 1995). The tissues of the preterm infant are more resistant to insulin, as demonstrated by the fact that after birth the preterm infant has higher plasma glucose and insulin levels than the term infant. Glucose production continues despite high glucose and insulin levels (Ogilvy-Stuart AL and K Beardsall 2010).

1.3.5.8 Complications and outcome

Hypoglycemia is a well-known risk factor for brain injury and poor neurodevelopmental outcomes in moderately preterm and term infants (Kerstjens JM et al. 2012; Tam EW et al. 2012). Using neonatal MRI, patterns of selective vulnerability after hypoglycemia was identified in the white matter and deep nuclear GM (Burns CM et al. 2008).

Less is known about adverse cerebral effects of hyperglycemia. Hyperglycemia in preterm infants has been associated with increased mortality rates (Hays SP et al. 2006; Kao LS et al. 2006; Heimann K et al. 2007; Alexandrou G et al. 2010), intraventricular hemorrhage (IVH) grades III to IV (Hays SP et al. 2006), bronchopulmonary dysplasia (Hays SP et al. 2006), sepsis (Kao LS et al. 2006), retinopathy of prematurity (Blanco CL et al. 2006; Mohamed S et al. 2013), and with increased lengths of hospital stay (Hall NJ et al. 2004). However, its associations to neurological morbidity are not well studied.

The Score for Neonatal Acute Physiology, Perinatal Extension, Version II (SNAPPE II) relies mainly on physiologic measurements for risk adjustment but takes baseline characteristics into consideration; it is a validated illness severity and mortality risk scoring system for newborn intensive care, which is simple, accurate, and robust across populations (Richardson DK et al. 2001). Glucose deviation, both above and below normoglycemic levels, is one of the factors it accounts for and impacts the score depending if the deviation merits careful monitoring or requires the physician to alter therapy to correct it. The inclusion of glucose levels in such a scoring system demonstrates the importance of glucose regulation in the newborn.

1.3.6 Sex

The male disadvantage with regards to perinatal mortality and morbidities is well known (Drevenstedt GL et al. 2008). Many studies in extremely preterm and extremely low birth weight populations have demonstrated increased mortality rates and in-hospital morbidity in males (Brothwood M et al. 1986; Hoffman EL and FC Bennett 1990; Stevenson DK et al. 2000; Lemons JA et al. 2001; Elsmen E et al. 2004; Tyson JE et al. 2008; Synnes AR et al. 2010). A recent study in preterm twins exploring sex-associated differences in perinatal outcomes concluded that in unlike-sexed twin pairs, very preterm males had higher respiratory morbidity than females. Male-male twins have higher respiratory morbidity and neonatal mortality than female-female twins (Steen EE et al. 2013). Another study using the National Institute of Child Health and Human Development Neonatal Research Network data, demonstrated that boys were more likely than girls to have adverse outcomes such as CP; male sex was an independent risk factor for Bayley Mental Developmental Indices <70 and neurodevelopmental impairment.(Hintz SR et al. 2006). In the EPICure study EPT boys had a group mean IQ score 10 points lower than the girls, and were at double the risk to have impaired cognitive function (Marlow N and H Budge 2005). In a study assessing adverse neurodevelopmental outcome among EPT children with no brain injuries, boys had a significantly higher prevalence of CP, and in multivariate models, significant associations were found between CP and male sex (Laptook AR et al. 2005).

Studies on sex differences in neonatal brain structure are few. Rose *et al.* (Rose J et al. 2009) demonstrated in extremely low birth weight infants that males had more MRI abnormalities and lower fractional anisotropy (FA) and higher mean diffusivity (MD) (described below) in the splenium of the CC and in the right posterior limb of the internal capsule; additionally, abnormal neurodevelopment was more common in males (Rose J et al. 2009). Sex differences in global intracranial volume, cortical GM, and cortical WM, as well as areas of local sexual dimorphism have been reported in full term neonates (Gilmore JH et al. 2007). In a recent study the same group showed that full term males had larger volumes in medial temporal cortex and rolandic operculum, and females had larger volumes in dorsolateral prefrontal, motor, and visual cortices. They concluded that androgen exposure and sensitivity had minor sex-specific effects on local GM volume, but did not appear to be the primary determinant of sexual dimorphism at this age (Knickmeyer RC et al. 2013).

1.4 INVESTIGATION OF THE PRETERM BRAIN WITH MRI

In this thesis MRI was used to investigate, qualitatively and quantitatively, brain abnormalities in extremely preterm infants at term equivalent age, and also to explore associations with neonatal risk factors and toddler age outcomes.

1.4.1 Magnetic resonance imaging

1.4.1.1 Background

Magnetic resonance imaging (MRI) is based on the phenomenon of nuclear magnetic resonance (NMR) and provides a non-invasive means for high-resolution brain imaging. In NMR the properties of different atomic nuclei spin systems is used to infer on molecular structure and their chemical environments. The most commonly used spin system in clinical MRI is protons (^1H) which is abundant in most tissues in water molecules (H_2O). Protons have two spin-states, conventionally denoted spin-up or spin-down. When placed in the strong magnetic field in the MR scanner the proton spin-states will have slightly different energy levels. This results in an unequal population of the spin-states, slightly favouring the lower energy state (spin-up). By applying a radiofrequency pulse (RF-excitation pulse) at a given frequency energy is absorbed within the spin-system lifting protons in the lower energy state (spin-up) up to the higher energy state (spin-down). The RF-excitation also creates a temporary phase-coherence among the spins. After the RF-pulse the spin-systems will return to its equilibrium state. The phase-coherence is lost fairly quickly (T2-relaxation process or spin-spin relaxation) and the absorbed energy is eventually lost to the surroundings (T1-relaxation process or spin-lattice relaxation). Protons in different molecular configurations or chemical environments will experience different T1- and T2-relaxation processes. This is utilized to create images with different tissue contrasts in order to display anatomical structures or pathologies. The image in a MR experiment is created by repeatedly applying RF-excitation pulses and recording the faint radiofrequency echoes that spin system can be stimulated to emit. T1-weighted images explore the differences in the T1-relaxation properties of the tissues, and are achieved by shortening the repetition time (TR) between successive RF-pulses while keeping the time between the RF-pulse and the readout of the signal/echo, the so called echo time (TE), short (to minimize differences in T2-relaxation). T2-weighted images explore differences in the T2-relaxation and by using a long TE while keeping the TR long (to minimize the differences in T1-relaxation). Tissues rich in fatty content, like the myelinated white matter, have a higher signal on T1-weighted images than tissues with more watery content, like the cerebral cortex. On a conventional T1-weighted image the signal difference between gray and white matter can be fairly subtle. Various techniques can be employed to increase the contrast to create MR images with large GM/WM contrast. These MR images can be used for tissue segmentation in quantitative volumetric studies. T2-weighted images are sensitive to display pathological processes, since these often cause a relative increase tissue water content, to detect e.g. edema and white matter lesions. The excellent soft tissue contrast provided by MRI makes it an ideal tool for investigating developmental changes in the preterm brain.

In the neonatal population quantitative MRI techniques can be used to define developmental trajectories and for comparisons to reference populations. Available tools for characterizing brain development and maturation by MRI make use of morphology, as well as the evolving MRI signal characteristics providing insight into the macroscopic and microscopic structural changes during this period.

1.4.2 Diffusion MRI

1.4.2.1 Background

Diffusion weighted MR imaging has been used to investigate subtle differences in cerebral growth and development in infants, children and adolescents born preterm or infants with very low birth weight.

In diffusion MRI the MR signal is sensitized to the water diffusion processes that are present in the tissue. Technically, two gradient lobes, so called diffusion encoding gradients, are applied between the RF-excitation and the signal readout. The diffusion gradients are sequential but have altered polarity and are separated in time. In stationary water molecules the effects from the diffusion gradients cancel each other. In water molecules that diffuse/move in the voxel between the first and the second gradient, which is the normal physiological case, the effects will not cancel each other resulting in an MR signal decay. The signal decay is proportional to the diffusion properties in the spatial direction in which the diffusion encoding gradients were applied. Diffusion processes are Brownian motions, which are random, thermally driven movements of molecules over time. The random Brownian motion was described in 1827 by a Scottish botanist, Robert Brown, who noted that pollen grains suspended in water were constantly moving in a random fashion. In isotropic diffusion the diffusion movement is equally possible in all directions and can be modeled as a sphere with the size corresponding to the amount of displacement in a given time. If the diffusion process is free the average displacement r of the molecules during a time t is given by the diffusion coefficient of the medium D in a simple equation:

$$r^2 = 6Dt$$

In the CSF water molecules move relatively freely and the diffusion can be considered isotropic. The organization of long neuronal axons in the WM preferentially inhibits water diffusion such that it appears relatively unhindered when the diffusion encoding is placed along the direction of a tract, but restricted when the gradient is placed perpendicular to the tract. This diffusion process is anisotropic and the geometrical analogy is a skewed ellipsoid where the longest axis represents the direction in which diffusion is greatest.

The diffusion coefficient D can be quantified with diffusion MR imaging. By relating one experiment with diffusion encoding (S) to one measurement with no diffusion encoding (S_0) the signal decay is given by the formula:

$$S = S_0 e^{-bD}$$

In the exponential factor, b is the so-called b-value and is a measure of the amount of the diffusion weighting from the diffusion gradient and is calculated from the diffusion MR imaging sequence. By re-arranging the equation the diffusion coefficient D can be estimated. The b-value defines the length scale in which the diffusion processes are studied. In WM typical axons sizes are 50-100 μm and b-values are around 1000 s/mm^2 are used. In a neonatal population lower b-values around 600-800 s/mm^2 are used due to the different tissue characteristics of the maturing brain. In vivo the motion of water molecules is not only a random motion but also driven by active transport or pressure gradient. Therefore, the measured diffusion coefficient is called the apparent diffusion

coefficient (ADC) to include these.

By repeating the measurement along several different spatial directions the three-dimensional diffusion properties in a voxel can be estimated. A simple model to describe the diffusion motion is the diffusion tensor model. This is geometrically the equivalent of describing the diffusion with an ellipsoid. The diffusion processes along each axis of the ellipsoid can be separated into directional and scalar components. The average diffusion distance along an axis is represented by a scalar magnitude known as the eigenvalue. The axes with the longest, middle and shortest magnitudes are denoted by the λ_1 , λ_2 and λ_3 eigenvalues, respectively, and the eigenvectors v_1 , v_2 and v_3 are their corresponding directional components. The average diffusivity across all three directions is known as the mean diffusivity (MD). Diffusivity along the principal axis is known as principal or axial diffusivity (AD), whilst the average of λ_2 and λ_3 is known as perpendicular or radial diffusivity (RD). AD is thought to reflect fiber coherence and structure of axonal membranes (Song SK et al. 2002), whereas RD is more related to the degree of myelination (Song SK et al. 2002; Cheong JL et al. 2009). Fractional anisotropy (FA) is a scalar value that captures the degree to which the tensor ellipsoid is isotropic or anisotropic. The FA is normalized such that it takes values from zero (purely isotropic) to one (purely anisotropic). Many factors influence the anisotropy and include axonal diameter and density, myelination, extracellular diffusion, inter-axonal spacing, and intravoxel fiber-tract coherence (Basser PJ and C Pierpaoli 1996). The tensor model represents a way of characterizing diffusion but is limited in regions of complex fiber organization, such as in regions where multiple fiber populations converge or cross. This is illustrated by the FA being large in a coherent fibre bundle but drops dramatically in areas of crossing fibres. This can make interpretation of FA in atypical brain tissues or after injury complicated (Groschel S et al. 2013).

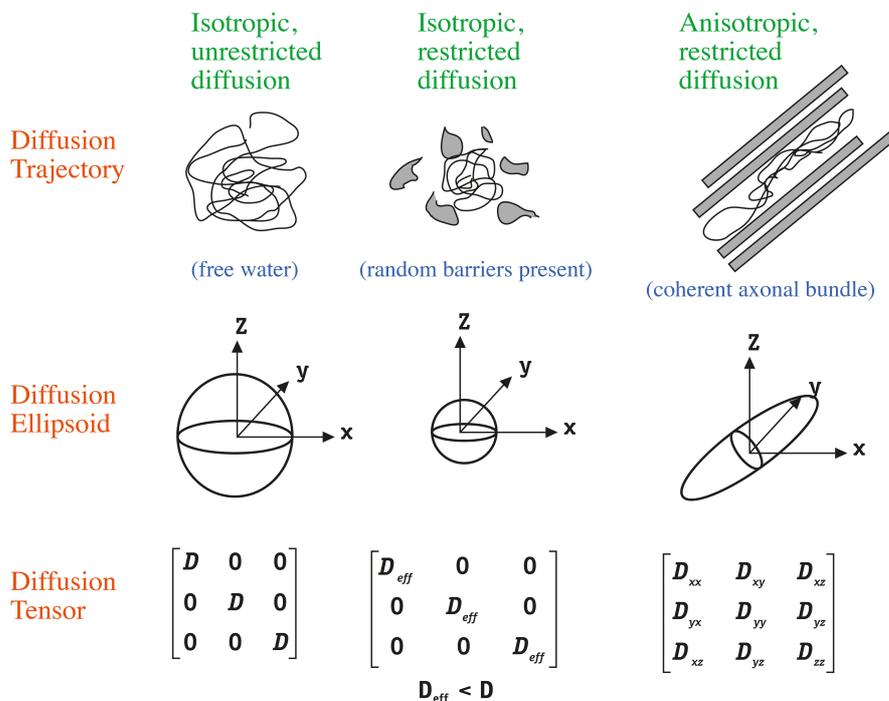


Figure 4. The diffusion ellipsoids and tensors for isotropic unrestricted diffusion, isotropic restricted diffusion, and anisotropic restricted diffusion are shown, adapted from P Mukherjee, 2008.

The influence of cerebral tissue on water movement enables diffusion MR imaging to be highly sensitive to microstructural changes, including those changes associated with premature development and disease. In neonates, FA has been found to increase, while MD, AD, and RD decrease with age in WM regions, likely due to increased fiber organization, axonal coherence, and preliminary myelination (Mukherjee P et al. 2002; Partridge SC et al. 2004; Dubois J et al. 2008; Aeby A et al. 2009; Shim SY et al. 2012).

1.4.2.2 Processing and analysis of Diffusion Tensor Imaging data

Comparative analysis of quantitative diffusion measures can be performed in different ways; regionally by delineating anatomical structure with region-of-interest, along fiber tracts delineated with fiber tractography algorithms, or on a whole-brain level, either with VBM-styled techniques in which diffusion measures in homologous voxels are compared or with Tract-Based Spatial Statistics where diffusion measures are mapped onto a common tract template and then compared.

In TBSS (Smith SM et al. 2004; Smith SM et al. 2006) the FA maps of all subjects are first aligned into a standard space using non-linear registration. The mean of all subjects' aligned FA images is created and then 'thinned' using non-maximum-suppression perpendicular to the local tract structure to create a mean FA skeleton that represents the centers of major tracts common to the group of subjects. A threshold for FA is then applied in order to include the major white matter tracts while suppressing peripheral tracts with low mean FA, high inter-subject variability and/or partial volume effects with grey matter.

Each subject's aligned FA data are then projected onto this skeleton perpendicular to the local tract direction, so that the projected FA values are taken from the centers of the tracts in the original FA image. This projection aims to resolve any residual alignment problems after the initial non-linear registration. The resulting data are then fed into voxelwise cross-subject statistics allowing an observer-independent multi-subject whole brain analysis.

Neonatal imaging data present challenges due to lower resolution and contrast of images, wide variations in brain size, complex changes in age-dependent brain maturation, and frequent motion artifacts, therefore modifications may be required to the default TBSS processing algorithm. An optimized protocol for neonatal data has been described by Ball et al. has been described to achieve more accurate spatial alignment of individual datasets (Ball G et al. 2010). This protocol includes two modifications to the TBSS default process. In order to improve global alignment between the neonatal FA maps, an initial low degrees-of-freedom linear registration is included in the processing, followed by a second registration to a study specific average FA map, which achieves accurate projection of the individual data on the FA skeleton.

1.4.3 Brain Tissue Segmentation, Volumetry and Voxel-based morphometry

An accurate segmentation method of MRI images plays an important role in quantifying the early brain development and subsequent quantitative analysis. In this respect, the infant brain presents challenges for tissue segmentation due to the MRI brain developmental pattern, which is different compare to adults. The neonatal brain shows a reversal of the normal adult pattern of MR intensities and an increased intensity inhomogeneity caused by the ongoing myelination (Paus T 2010). Thus, the

intensities of various tissues may have different patterns to those in adults and consequently the behavior of the segmentation algorithm is likely to be unpredictable. In addition, the presence of significant natural and pathological anatomical variability might affect the brain tissue segmentation. In relation to this, several aspects have to be considered. Firstly, the use of MRI images of high quality that has a key role in the segmentation processes. It is known that motion artifacts may result in poor spatial resolution. Secondly, the use of adequate reference data for spatial normalization and segmentation. The use of default adult or pediatric template to segment infant's brain data results in misclassifications as demonstrated by (Altaye M et al. 2008; Wilke M et al. 2008). Consequently, for minimizing this problem, it is important to choose, atlas subjects with an age similar to the test subject. Finally, the use of recent advances in image segmentation and registration processes that enable more accurate segmentation. The new segmentation toolbox of the SPM v8 software for automatic segmentation is an extension of the default-unified segmentation (Ashburner J and KJ Friston 2005). The algorithm is essentially the same, except for a different treatment of the mixing proportions, the use of an improved registration model, an extended set of tissue probability maps, which allow a different treatment of voxels outside the brain, and a more robust initial affine registration. By using this algorithm, we could use the deep grey matter, cerebellum and brainstem in addition to cortical GM, WM and cerebrospinal fluid tissue probability maps.

1.4.4 Voxel-based morphometry-DARTEL

Voxel-based morphometry is an automated procedure for quantifying GM and WM regional changes through a voxel-by-voxel analysis of MRI data (Ashburner J and KJ Friston 2000) and allows between-groups comparisons. The VBM-Diffeomorphic Anatomical Registration Through Exponentiated Lie algebra (DARTEL) algorithm analyses improve inter-subject registration (Ashburner J 2007), which is indispensable in preterm samples. The DARTEL algorithm is based on a more sophisticated registration model that notably improves the realignment of small inner structures (Yassa MA and CE Stark 2009).

1.5 NEUROLOGICAL AND DEVELOPMENTAL OUTCOME FOLLOWING PRETERM BIRTH

1.5.1 Neurological and neuromotor outcomes

Children born very preterm (born <32 weeks of gestation) or extremely preterm (born <28 weeks of gestation) are at risk of developing neuromotor impairments and at risk of atypical motor development despite advances in perinatal and neonatal care (Saigal S and J Tyson 2008). Neuromotor impairments range from major impairments, i.e. Cerebral Palsy (CP) to minor neurological dysfunction. For preterm and very low birth weight (<1500g birth weight) children, the Surveillance of Cerebral Palsy in Europe study showed a CP prevalence of 39.5 (28.6–53.0) per 1000 live births in 1996 (Platt MJ et al. 2007). For western Sweden, Himmelmann *et al* (Himmelmann K et al. 2010), calculated for the birth years 1998-2002 a gestational age-specific prevalence of CP for <28 gestational weeks of 55.6 per 1000 live births, 43.7 for 28-31 weeks, 6.1 for 32-36 weeks and 1.43 per 1000 for >36 weeks. Himpens *et al* (Himpens E et al. 2008) conducted a meta-analysis, which included 26 studies, and concluded that the prevalence of CP decreases significantly with increasing GA category: 14.6% at 22 to 27 weeks' gestation, 6.2% at 28 to 31 weeks, 0.7% at 32 to 36 weeks, and 0.1% in term infants.

Several systematic reviews and meta-analyses (e.g. de Kievit *et al* (de Kieviet JF *et al.* 2012); Williams *et al* (Williams J *et al.* 2010)) have shown that preterm and very low birth weight children who do not have CP have a high prevalence of minor neurological dysfunction and motor skill impairment. In the EPIPAGE study (Arnaud C *et al.* 2007), which follows a large cohort of preterm children born <33 weeks in France, 44% of the children had minor neurological dysfunction at the age of 5 years and this was associated with learning difficulties. DeKievit *et al* (de Kieviet JF *et al.* 2012) found on analysis of 41 studies that children born at gestational age ≤ 32 weeks or with a birth weight of ≤ 1500 g had significantly lower scores on variety of motor tests compared to term born peers. Williams *et al* (Williams J *et al.* 2010) established in a systematic review of the literature on school age children born at <37 weeks of gestation that 40.5% has mild-moderate problems and 19% had moderate problems on standardized tests of neuromotor function.

While neuroimaging correlates for CP in preterm children have been established, there is still very little information available on potential neuroanatomical correlates in preterm children with minor neurological dysfunction. For CP, the European CP study, white matter damage including cystic PVL and periventricular hemorrhage, was the most common neuroimaging finding in about 80% of infants with CP born < 34 weeks of gestation. Comparable data have been described in a systematic review which indicates, that preterm children with CP 90% have focal white matter injury, (including PVL) and large hemorrhagic lesions (Krageloh-Mann I and V Horber 2007).

1.5.2 Neurodevelopmental, cognitive and behavioral outcomes

Neurodevelopmental, cognitive and behavioral outcomes have been extensively studied in preterm children (for review see e.g. Saigal *et al* (Saigal S and LW Doyle 2008)), A close correlation between overall cognitive function with gestational age and birth weight has been observed (Bhutta AT *et al.* 2002; Kerr-Wilson CO *et al.* 2012). Even in the context of normal overall cognitive function, specific cognitive deficits, behavioral difficulties, and poor academic achievement are frequently seen (for review see e.g. Aaroundse-Moens *et al* (Aarnoudse-Moens CS *et al.* 2009)). However, large and long-term outcome studies that focus on extremely preterm children are few.

The EPICure, a series of studies of survival and later health in infants born at less than 26 weeks of gestation in the UK and Ireland, showed that the Mental Developmental Index in children born < 26 weeks' gestation was on average 1 SD below the published norms mean (84 ± 12). 10% of those without severe neuromotor or sensory and communication problems, were classified as having a severe cognitive disability (equivalent to Bayley scores under 55) (Wood NS *et al.* 2000). At 11 years of age, the extremely preterm children were more than three times more likely to have a psychiatric disorder than classmates, the risk for attention-deficit hyperactivity disorder was significantly increased, there was an increased risk for anxiety disorders as well as for autism spectrum disorders (Johnson S *et al.* 2010).

A study of 8 year old children with extremely low weight at birth (<1000 g) in the USA, showed significantly higher mean Symptom Severity Scores for the inattentive, hyperactive, and combined types of attention-deficit hyperactivity disorder in the children with extremely low birth weight when compared to term born controls (Hack M *et al.* 2009). A Canadian study of a five-year cohort of children with weight at birth <800 g showed that the incidence of cognitive impairment at school entry level

was 21% (Synnes AR et al. 2010). Saigal et al studied school difficulties in a cohort of extremely low birth weight teenagers and term born controls. This study demonstrated that approximately 70% of those with extremely low birth weight had school difficulties a significant proportion required special educational assistance and/or had repeated a grade. Problems were apparent even in children without neurosensory impairments and normal IQ (Saigal S et al. 2000).

Recently, the Swedish national study, investigating neurodevelopmental outcome in children born extremely preterm at 2.5 years corrected age demonstrated that moderate and severe cognitive impairment was present in 5.0% and 6.3% of the cohort, respectively. Moderate or severe mental developmental delay was seen in 20% of children born extremely preterm (Serenius F et al. 2013)(Serenius F et al. 2013)(Serenius F et al. 2013)(Serenius F et al. 2013)(Serenius F et al. 2013) Neurodevelopmental Outcome in Extremely Preterm Infants at 2.5 Years After Active Perinatal Care in Sweden).

1.5.3 MRI at TEA and relation to neurodevelopment

A number of studies have shown that neonatal MRI findings can aid in prediction of later neurodevelopmental outcomes in children born preterm.

In a seminal paper, abnormal findings on visual inspection of MRI at TEA in very preterm infants strongly predicted adverse neurodevelopmental outcomes at two years of age (Woodward LJ et al. 2006). On the other hand, there have been conflicting reports on the importance of diffuse excessive high-signal intensities (DEHSI), without obvious brain injuries (Dyett LE et al. 2006; Skiold B et al. 2012) with regards to developmental outcome.

Various studies have explored the relationship between MRI diffusion measurements at TEA and neurodevelopmental outcome. For example, Van Kooij *et al* showed that cognitive scores were correlated with FA in the CC suggesting that MRI diffusion measures at TEA have the potential to be used as a biomarker for subsequent neurodevelopment (van Kooij BJ et al. 2012).

Associations between WM volumes in the right sensorimotor and right mid temporal regions and Mental Development Indices on the Bayley Scale of Infant Development-II at 2 years corrected age have been reported (Peterson BS et al. 2003). Similarly, cortical and subcortical GM reduction were demonstrated in preterm infants who later had moderate to severe disability (Inder TE et al. 2003; Inder T et al. 2005). Moreover, reduced total cerebral tissue volumes in the premotor, sensorimotor and parieto-occipital regions with increases in the cerebral spinal fluid were significantly correlated to decreasing task performance on an object working memory task (Woodward LJ et al. 2005).

2 AIMS

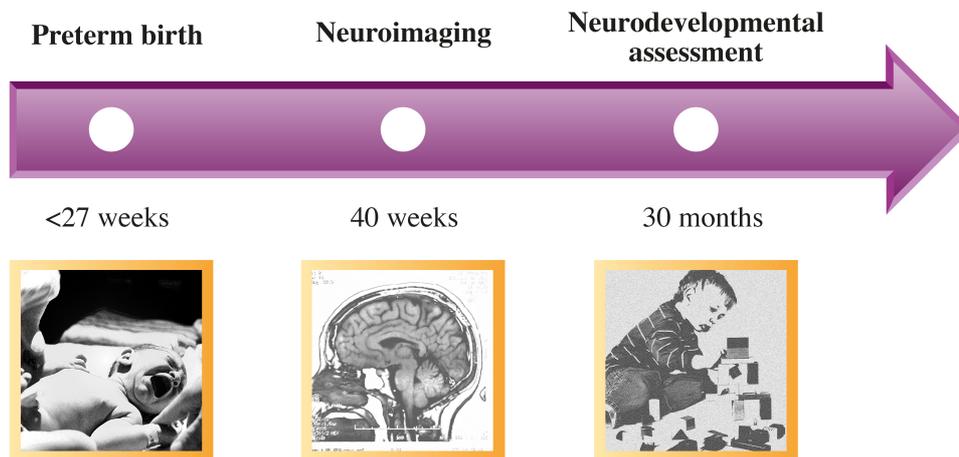
The overall aim of the work compiled in this thesis was to qualitatively and quantitatively study brain abnormalities of extremely preterm infants at term equivalent age (TEA) and to investigate associations with neonatal risk factors and with infant and toddler age outcomes.

The specific aims were:

- i. To compare white matter microstructure using MR diffusion measures (FA and MD) in major white matter tracts of EPT infants without focal brain lesions to a term-born control group (Paper I).
- ii. To explore the respective contributions of immaturity at birth and neonatal risk factors to variation in white matter diffusion measures at TEA within the group of EPT infants (Paper I).
- iii. To study the incidence of early hyper- and hypoglycemia during the first week after birth in a cohort of EPT infants (Paper II).
- iv. To determine whether hyperglycemia during the first day of life in EPT infants is associated with increased mortality rates and with overt cerebral injury as assessed with structural MRI of the brain, at TEA (Paper II).
- v. To explore associations between early hyperglycemic exposure, white matter microstructure and brain tissue volumes in EPT infants scanned at TEA (Paper III).
- vi. To investigate sex differences in neurological and developmental outcome in children born EPT and to explore associations with brain structure as assessed with conventional structural MRI and diffusion MRI at TEA (Paper IV).

3 PARTICIPANTS AND METHODS

3.1 STUDY DESIGN



3.2 ETHICAL CONSIDERATIONS

The Karolinska University Hospital ethics committee granted approval to all studies included in this thesis. Informed consent was obtained from all parents of the participating infants.

Results from individual MRI examinations were given to the parents by the neonatologist in the ward, as part of the clinical follow up of the EPT infants. There were no adverse events reported during the neuroimaging.

3.3 PARTICIPANTS

3.3.1 Preterm Infants

Infants born in the Stockholm region with a GA less than 27 weeks were eligible for the studies.

The four-year study period, in studies I and III, included infants born from January 1st 2004 to December 31st 2008. During this period a total 180 EPT infants underwent MRI at TEA and were eligible for inclusion in studies I and III. Out of these 125 underwent diffusion MR imaging.

Study II, included infants born from January 1st 2004 to December 31st 2006. During this period 94 preterms underwent MRI at TEA and had documented glucose levels and were eligible for inclusion.

Study IV included infants born from January 1st 2004 to March 31st 2007. A total of 108 preterm infants underwent MRI at TEA.

3.3.1.1 Medical exclusion criteria

Exclusion criteria were malformations, chromosome aberrations and congenital infections (n=8). A pair of twins with congenital cytomegalovirus infection, one infant with Down Syndrome, one infant with oesophageal atresia, one with myelomeningocele, one with hemophagocytic lymphohistiocytosis, one with intracranial vascular malformations, and one infant with cleft lip and palate were excluded from the study. Four families moved away from the Stockholm region and nine infants parents declined participation in the MRI examination. Another infant was too unstable for MRI at TEA.

Infants with brain abnormalities such as focal lesions (IVH grades III-IV and cystic PVL) and marked ventricular dilatation on visual inspection of T1-w and T2-w images were excluded from Studies I, III and IV (n=10).

3.3.1.2 Imaging exclusion criteria

In the studies with quantitative assessments of MRI using DTI and VBM, (Studies I, III and IV) data sets with suboptimal diffusion data due to movement, pulsation artefacts and incomplete coverage of the brain (n=74) and suboptimal 3D T1-w data sets for VBM due to blurring of white and grey matter interfaces motion artefacts (n=15) were excluded.

3.3.2 Term born control infants

3.3.2.1 Imaging Controls

Twenty-one healthy term-born control infants delivered after planned caesarean section were recruited from the maternity ward. Controls underwent imaging at the same postmenstrual age as the preterm infants and were scanned on the same scanner according to the same protocol. Seven of these infants' datasets were removed from further consideration due to movement artefacts in Study I.

3.3.2.2 Neurodevelopmental Controls

For comparison of outcome data, a control group of 85 term-born healthy children, matched for maternal residential area, age and sex underwent neurodevelopmental assessment. This group, however, did not undergo MRI.

3.4 METHODS, AN OVERVIEW OF SUBJECTS, METHODS AND OUTCOME

STUDY	PERIOD	SUBJECTS	METHODS	INCLUSION CRITERIA	EXCLUSION CRITERIA	RISK FACTOR	OUTCOME
I	2004-08	EPTS (N=58), IMAGING CONTROLS (N=14)	TBSS, ROI	GOOD QUALITY DTI	FOCAL LESIONS, DILATED VENTRICLES, MEDICAL REASONS	MECH. VENT., BPD, LOW GA	BRAIN MICROSTRUCTURE
II	2004-06	EPTS-LIVING (N=94) EPTS- DECEASED (N=19)	WM SCORE, GLUCOSE SCORE	GOOD QUALITY MRI, GLUCOSE DOCUMENTATION	DEATH BEFORE TERM-AGE, MEDICAL REASONS	HYPERGLYCEMIA	WM REDUCTION, DEATH
III	2004-08	EPTS (N=38)	TBSS, ROI, SEGMENTATION, VBM, GLUCOSE SCORE	GOOD QUALITY DTI AND STRUCTURAL MRI. GLUCOSE DOCUMENTATION	FOCAL LESIONS, DILATED VENTRICLES, MEDICAL REASONS	HYPERGLYCEMIA	BRAIN MICROSTRUCTURE & VOLUMES
IV	2004-07	EPTS (N=27-29), NEURODEV. CONTROLS (N=91)	WM SCORE, TBSS, SEGMENTATION, VBM	GOOD QUALITY DTI AND STRUCTURAL MRI	FOCAL LESIONS, DILATED VENTRICLES, MEDICAL REASONS	SEX	NEUROLOGICAL, BSD-II, AND BRAIN MORPHOLOGY

Table 1. An overview of studies included in this thesis

3.4.1 Magnetic Resonance Imaging

3.4.1.1 Conventional imaging

All infants were scanned at the Astrid Lindgren Children’s Hospital in Stockholm, Sweden. Data were acquired on a Philips Intera (Philips Intera, Philips Medical, Best, Netherlands) 1.5 Tesla scanner with a 6-channel SENSE receive-only head coil. Sequence parameter details may be found in Table 2. Noise reduction and hearing protection was provided with individually molded earplugs (*Affinis Dental Putty Soft, Forsberg Dental, Sweden, 10-20 dB*) and neonatal (*Mini-Muffs Natus Medical Inc, San Carlos, CA, 7 dB*) and pediatric earmuffs (*Bilsom Junior, Bacou-Dalloz Nordic, Sweden, 15-32 dB*). Additional reduction of scanner noise was obtained by a custom-made sound dampening “hood” attached to the upper half semicircle of the magnet bore reducing the noise level with up to 24 dB (Nordell A et al. 2009). A physician, experienced in MRI procedures, monitored all infants throughout the imaging session. Initially the infants were lightly sedated using chloral hydrate 30 mg/kg orally or rectally. Later the infants were scanned during natural sleep, including the controls.

The MRI Protocol	
<ol style="list-style-type: none"> 1. Survey (00:50) 2. Reference scan (00:30) 3. Sagittal T2-w (02:30) 4. Sagittal T1-w (02:50) 5. Axial T2-w (01:00) 6. 3D T1-w (04:30) 7. Axial T1-w IR (02:55) 8. Axial T2*w (02:10) 9. Axial fMRI (10:00) 10. Axial DWI (02:40) 	
<p>Sagittal T2-w TE/TR/Flip = 100ms/5000ms/90deg. Slices = 19 ETL = 16 FOV = 180 Voxel size = 0.7mm x 0.7mm x 3.0mm SENSE = 2</p> <p>Sagittal T1-w TE/TR/Flip = 9ms/600ms/90deg. Slices = 24 ETL = 3 FOV = 180 Voxel size = 0.7mm x 0.7mm x 4.0mm SENSE = 2</p> <p>Axial T2-w TE/TR/Flip = 100ms/5000ms/90deg. Slices = 22 ETL = 16 FOV = 180 Voxel size = 0.7mm x 0.7mm x 4.0mm SENSE = 2</p> <p>3D T1-w TE/TR/Flip = 5ms/40ms/30deg. Slices = 22 FOV = 170 Voxel size = 2.0mm x 2.0mm x 2.0mm SENSE = 1.8</p>	<p>Axial T1-w IR TE/TR/Flip = 15ms/3500ms/90deg. Slices = 25 FOV = 180 IR = 400ms Voxel size = 2.0mm x 2.0mm x 4.0mm SENSE = 1</p> <p>Axial T2*w TE/TR/Flip = 23ms/586ms/18deg. Slices = 20 FOV = 180 Voxel size = 2.0mm x 2.0mm x 5.0mm SENSE = 1.5</p> <p>Axial fMRI TE/TR/Flip = 50ms/2000ms/80deg. Slices = 28 FOV = 180 Voxel size = 2.8mm x 2.8mm x 4.5mm Volumes = 300</p> <p>Axial DWI TE/TR/Flip = 74ms/7500ms/90deg. Slices = 28 FOV = 180 Voxel size = 1.4mm x 1.4mm x 2.2mm SENSE = 1.5 B = 700 Directions = 15 (OVERPLUS)</p>

Table 2. Sequences and parameters of the Neo-BIG (Neonatal Brain Imaging Group) protocol. Abbreviations: ETL: Echo Train Length, FOV: field of view, IR: inversion recovery, MRI: magnetic resonance imaging, TE: echo time, TR: repetition time, T1-w: T1-weighted, T2-w: T2-weighted

3.4.1.2 Scoring System for Conventional MRI

Conventional structural scans (T1- and T2-w images) were assessed by a neuroradiologist. Qualitative WM abnormalities were defined based on a previously published scoring system (Inder TE et al. 2003). This system assessed five separate items: abnormal white matter signal, reduced white matter volume, cystic changes, myelination/thinning of the corpus callosum and ventricular dilatation. WM abnormalities were further classified by the composite scores of these five categories (ranging from 5-15) into: no WM abnormalities (score 5-6), mild WM abnormalities (score 7-9), moderate WM abnormalities (score 10-12) or severe WM abnormalities

(score 13-15). The inter-observer agreement rate for WM abnormalities was 95,5% (Skiold B et al. 2010).

GM was graded similarly for 3 variables: abnormalities in cortical GM signal, maturity of cortical gyration rated with standard gyral models, and size of the subarachnoid space. Composite GM scores then classified infants as having i) normal or ii) abnormal gray matter.

3.4.1.3 *Automatic Brain Segmentation and Voxel-based morphometry-DARTEL*

The prior manual steps included reorientation of the original T1-w images in the plane of anterior-posterior commissures and removal of non-brain-tissue components using the Brain Extraction Tool (Smith SM 2002). Images were then segmented into tissue classes using unified segmentation (Ashburner J and KJ Friston 2005) as implemented in the “new segment” option of the SPM v8 software, (Wellcome Trust Centre for Neuroimaging, Centre for Neuroimaging, UCL, London, UK, running on MATLAB v7.5, MathWorks, Natick, MA). For guiding segmentation, we used tissue probability maps from preterm infants scanned at term age (Kuklisova-Murgasova M et al. 2011). The segmented brain tissues were spatially normalized using DARTEL (Ashburner J 2007). Finally, all images were modulated and smoothed with a full width at half-maximum of 3-mm Gaussian kernel. Using these smoothed brain tissue images we conducted the statistical analyses as outlined below. Global brain tissue volumes in cm³ were extracted from the segmented/normalized/modulated images of each subject with the Easy Volume toolbox (Pernet C et al. 2009).

3.4.2 **Diffusion MRI**

Data pre-processing and analysis was performed using FMRIB’s software library (FSL version 4.1; Oxford Centre for Functional MRI of the Brain (FMRIB), UK; <http://www.fmrib.ox.ac.uk/fsl/>). Image artifacts due to eddy current distortions were minimized by registering the diffusion images to the b0 images. Non-brain-tissue components were removed using the Brain Extraction Tool BET (Smith SM 2002). Fractional anisotropy maps were calculated using the FMRIB’s Diffusion Toolbox v.2.0 (FDT) (Smith SM et al. 2004). After calculation of the FA map for each subject, a voxel-wise statistical analysis of the FA data using Tract-Based Spatial Statistics v1.2 was implemented. Brain extraction was performed using DTI post-processing calculated values for the directional preference of water diffusion (fractional anisotropy, FA), the mean displacement of water molecules (apparent diffusion coefficient, ADC), the principal eigenvalue of the diffusion tensor (axial diffusivity, AD), and the average of the second and third eigenvalues of the DT (radial diffusivity, RD).

3.4.2.1 *Tract-Based Spatial Statistics analysis*

Group-wise multi-subject whole brain automated analyses were performed to investigate differences in diffusion measures between groups. Data were processed and analyzed using FMRIB’s Diffusion Toolbox (FDT version 2.0) and TBSS version 1.2 in FSL (version 4.1.4). The optimized protocol for neonatal data sets as described by Ball *et al* (Ball G et al. 2010) was implemented to achieve more accurate spatial alignment of individual datasets. The mean of all aligned FA images was then created and thinned to generate a skeletonized mean FA image (threshold > 0.2) to reflect common tracts across all subjects. Mean diffusivity, AD, and RD were

processed similarly to FA data with the exception that FA images were used to drive the nonlinear registration and skeletonization stages.

3.4.2.2 Region-of-interest analyses

In addition to whole brain analyses, Region-of-Interest (ROI) analyses were performed. These were mainly carried out to confirm findings from whole brain analysis in the group comparison analyses, and also to assess the strength of correlations between diffusion measures and neonatal risk factors.

All measurements were obtained with an in-house developed software based on the b=0 and directional colored FA images for each subject. Masks were placed on voxels of the FA skeleton where significant differences between groups were seen in the whole brain analysis. The regions were identified using an MRI atlas of human white matter atlas (Mori S WS, van Zijl PCM, Nagae LM 2005). The ROIs were automatically set at the same location for each participant's registered FA and MD maps. Group comparisons were carried out using independent samples Student t-test with Bonferroni correction.

3.4.3 Neurodevelopmental follow up

3.4.3.1 Neurological examination

At 30 months corrected age, infants underwent a neurological examination by an experienced paediatric neurologist assessing movements, posture, reflexes and muscular tone. Infants were then classified into three groups: 'normal' when they had an entirely normal neurological status, 'abnormal' when neurological signs of cerebral palsy were present as defined by the Surveillance of Cerebral Palsy in Europe (2000), SCPE, and a third group of infants exhibiting 'unspecific signs', such as asymmetry of muscular tone or reflexes, muscular hypotonia, or muscular hypertonia but not fulfilling the SCPE criteria.

3.4.3.2 Bayley Scales of Infant and Toddler Development – III

Both the BSID-III and the neurological examination were performed on the same day. The BSID-III assesses the development of infants and toddlers, 1-42 months of age. It consists of a series of developmental play tasks and takes between 45 - 60 minutes to administer. Raw scores are converted to scale scores and to composite scores. These scores are used to determine the child's performance compared with U.S. standardization norms taken from typically developing children of their age, with a mean of 100 and a standard deviation of 15 (Bayley N 2005).

The scales of the BSID-III used for our study were: the motor (fine and gross skills), language (receptive and expressive communication), and cognitive scales. The social-emotional and adaptive function scales were not utilized in our research.

3.4.4 Glucose monitoring, documentation and scoring systems

Blood and plasma glucose level values were retrieved retrospectively from the clinical charts for the first week of life. Hyperglycemia was defined as plasma glucose levels of >8.3 mmol/L and hypoglycemia as plasma glucose levels of <2.6 mmol/L. Infants were identified as having hyperglycemia, hypoglycemia, or both, according to these criteria,

for each day of the week.

3.4.4.1 *Glucose monitoring protocol*

The clinical protocol recommended glucose sampling several times per day for the first days of life. Urinary glucose levels were checked at each micturition. Glucose monitoring was less stringent on subsequent days if glucose levels were stable (3– 8 mmol/L), if the infant was in clinically stable condition, and if there was no glycosuria. The number of glucose values for each day differed and, from 1 to 6 days of life, there were increasing numbers of infants without any measured values.

3.4.4.2 *Glucose Documentation*

Glucose readings were performed and documented by the nursing staff by using the HemoCue 201 (HemoCue, Inc, Lake Forest, CA) glucose method (Banauch D et al. 1975). Blood samples were obtained from umbilical or peripheral arterial lines infused with saline solution only or from peripheral veins.

3.4.4.3 *Hyperglycemic Scoring Systems*

To grade the hyperglycemic load for the first week of life, a scoring system was used (Heimann K et al. 2007). Initially the infants were categorized according to the number of days with hyperglycemic levels. The relative number of hyperglycemic episodes, indicative of hyperglycemic exposure, was calculated separately for the first 24, 48 h and week for each individual using the following equation:

Relative number of hyperglycemic episodes (24, 48 h or first week) = [Number of hyperglycemic measurements per subject (24, 48 h or first week) / Total number of measurements per subject (24, 48 h or first week)] x the maximal total number of measurements in one of all subjects in the specific time period (24, 48 h or first week).

The infants were then categorized into groups (groups I–III) on the basis of the relative number of hyperglycemic episodes, as follows: group I, no relative plasma glucose levels of ≥ 8.3 mmol/L; group II, 1 to 3 relative plasma glucose levels of ≥ 8.3 mmol/L; group III, ≥ 4 relative plasma glucose levels of ≥ 8.3 mmol/L.

3.5 STATISTICAL ANALYSIS

Statistical analyses were performed with PASW Statistics[®] software (*SPSS Inc, Chicago, IL*) version 19.0 in Study I, version 17.0 in Study II, version 20.0 in Study III and version 21.0 in Study IV.

Continuous variables are presented as mean and SDs, and categorical variables as frequencies and percentages. Normally distributed continuous data were analyzed with Student's t-test. For non-normally distributed data, the Mann-Whitney U test was applied. Categorical data were analyzed with Pearson's chi-square or Fisher's Exact test where applicable.

In Study I, the group-wise TBSS analyses were performed using Randomize (Nichols TE and AP Holmes 2002). Results underwent cross-subject statistics with Family-Wise Error correction for multiple comparisons using cluster-based thresholding ($t > 3$; $p < 0.05$). Group comparisons of measures from the ROIs were carried out using

independent samples Student t-test with Bonferroni correction. Within the preterm group, associations between diffusion measures and neonatal risk factors were first explored with correlation analyses in TBSS. Linear regression analyses within TBSS were then performed with the variables that were significantly correlated with FA or MD in the univariate correlation analyses in TBSS, to examine the contribution of those individual variables to explaining variation on diffusion measures. FA and MD were extracted from ROIs placed in the regions identified in the group-wise analyses in TBSS; and multivariate regression analyses were performed to assess the strength of correlations between diffusion measures and neonatal risk factors.

In Study II, repeated-measures analysis of variance was used to determine the differences in glucose variability for the whole week among survivors and deceased infants. Multivariate linear regression was performed to examine the associations between the outcome variables (primary outcome of death and secondary outcome of MRI abnormalities) and clinical and demographic explanatory variables selected on the basis of earlier research and results of univariate analyses. A receiver operating characteristic (ROC) curve for plasma glucose levels during the first 24 hours after birth, with respect to death was used to identify the most sensitive and specific glucose cut-off level for defining hyperglycemia.

In Study III, the group-wise TBSS statistical analyses were performed using Randomize (Nichols TE and AP Holmes 2002). Analyses were adjusted for CRIB score and sex. Associations between diffusion measures and hyperglycemia measures were explored with regression analyses in TBSS. In addition, multivariate regression analyses were performed using FA extracted from those ROIs (placed where significant differences were detected with TBSS) to further explore associations between hyperglycemia and diffusion measurements.

To assess differences on global volumetric measures between hyperglycemic and normoglycemic groups a general lineal model analysis (multivariate) was performed with GM and WM brain volumes as dependent variables and group status and sex as the independent factor. CRIB score was used as a covariate.

In the VBM-DARTEL analyses, independent samples Student t-test group comparisons were performed to evaluate sex differences, and simple regression analyses were performed in boys and girls separately to test for possible relationships at voxel level between brain volumes and developmental scores. Family-Wise Error correction ($p < 0.05$) was applied.

In Study IV, neurological status was compared between boys and girls using Pearson's χ^2 test, and scores from the BSID-III were compared using Students t-test. Differences in outcomes between boys and girls in relation to MRI findings were investigated through two sets of analyses; first in all EPT infants with available MRI data, and then in those without moderate-severe WM abnormalities, abnormalities in the GM, nor abnormalities in the cerebellum. The contribution of perinatal characteristics and MRI findings on the variance in BSID-III outcomes was investigated with linear regression models including sex, number of days on continuous positive airway pressure (CPAP), abnormalities in the cerebellum and delayed myelination on MRI. Possible relationships between global brain volumes and BSID-III scores, calculated separately for boys and girls, were investigated with Pearson's correlations.

Differences in global volumetric measures between boys and girls were assessed with a general lineal model analysis (multivariate) with brain volumes as dependent variables

and sex as the independent factor, controlling for total intracranial volume as covariate (all brain tissues, including CSF). Regional brain volume differences between boys and girls were performed with t-tests in VBM-DARTEL; and multiple regression analyses, adjusted for ICV, were performed in boys and girls separately to test for possible relationships at voxel level between brain volumes and developmental scores. Family-Wise Error correction ($p < 0.05$) was applied.

Group-wise TBSS analyses between boys and girls were performed using Randomize (Nichols TE and AP Holmes 2002). Results underwent cross-subject statistics with Family-Wise Error correction ($p < 0.05$) for multiple comparisons using Threshold-Free Cluster Enhancement.

4 RESULTS AND DISCUSSION

4.1 SURVIVAL AND NEONATAL MORBIDITIES

Between January 1st, 2004 and December 31st, 2006, 143 EPT infants with a gestational age < 27 weeks were born alive (i.e. with a measureable heart rate immediately after birth) in the Stockholm region. The survival rate in the cohort that formed the basis of the work described in this thesis was 83%, which is slightly higher, but comparable with the national EXPRESS Study 2004-2007 (Fellman V et al. 2009) that reported a 70% one-year survival rate of infants born alive at 22 to 26 weeks. The slightly higher survival rates presented in our study are due to the calculation based on slightly more mature infants born with GA between 23 to < 27 weeks of gestation. In comparison, survival rates at discharge from other studies in the preterm population were lower (Costeloe K et al. 2000; Tommiska V et al. 2001; Vanhaesebrouck P et al. 2004; Markestad T et al. 2005; Costeloe K 2006; Draper ES et al. 2009). Survivors had significantly higher GA and birth weight, and significantly lower Clinical Risk Index for Babies (CRIB) scores than did infants who died before TEA (Table 1, Paper II). Moreover, deceased infants (n=19, 13%) suffered from higher rates of major neonatal morbidities, such as grade III or IV IVH and necrotizing enterocolitis. The median age of death was 9 days (range: 3–30 days). Eight infants died during the first week after birth. The main causes of death were grade IV IVH (12 out of 19 infants), sepsis (9 out of 19 infants), and respiratory/cardiac failure (10 out of 19 infants), with an overlap of diagnoses in 8 out of the 19 cases.

Of the surviving infants, over the 4-year period (2004 - 2008), the infants with GA < 25 weeks had higher CRIB scores ($p = 0.001$), needed mechanical ventilation more frequently ($p = 0.015$), and received postnatal steroids more frequently ($p = 0.031$) compared with those born after 25 weeks GA (Table 1, Paper I). The overall prevalence of BPD in our study was 29%, in accordance with the 25% presented in the national study (Fellman V et al. 2009).

4.2 BRAIN IMAGING FINDINGS IN EXTREMELY PRETERM INFANTS AT TERM EQUIVALENT AGE

4.2.1 Findings on visual inspection of conventional structural MRI (Papers I, IV)

According to the white matter composite score, 14% of the whole cohort had moderate-severe WM abnormalities on MRI at TEA, 40 % had no WM abnormalities, 46 % had mild WM abnormalities, 2% had moderate and 12% had severe WM abnormalities (Skiold B et al. 2010). Abnormal GM was seen in 8% of the infants.

Over the four-year period (2004 - 2008), out of the 180 examined MRI data sets, 7 were excluded due to the presence of dilated ventricles (due to enlargement by the accumulated blood or when bleeding extended into the brain tissue around the ventricles) and 6 developed BPD. Three preterm data sets were excluded due to the presence of focal lesions (Figure 1, Paper I). From the remaining EPT infants included in the final analyses, low grade IVH I and II, was found in 21% of the preterms (Paper I). The prevalence of IVH reported in our study is twice as high as that reported in the

EPIcure study, however they reported findings only for IVH grade II (Wood NS et al. 2003). Our data are comparable to findings from the EPIPAGE study, in which, a more mature cohort in regards to GA was examined (Larroque B et al. 2003). Periventricular leukomalacia was found in 5% of the EPT infants of whom 2 developed BPD (Paper II), a finding in accordance with the 5.6% prevalence reported in the national EXPRESS study (Fellman V et al. 2009) and lower than the 10% presented in the EPIPAGE study in the subgroup of infants with comparable gestational ages as the ones in our cohort (Larroque B et al. 2003).

4.2.2 Diffusion MRI findings (Paper I, III, IV)

4.2.2.1 MRI diffusion measures – comparison between preterm and term controls

In the TBSS analyses, the EPT infants had significantly lower FA bilaterally in the frontal WM, corona radiata (CR), the centrum semiovale (CSO), the genu and splenium of the CC, the inferior longitudinal fasciculus (ILF), and the left external capsule (EC) compared to term controls (Figure 3, upper panel, Paper I). No regions were found where EPT infants had significantly higher FA than the controls.

The present study was designed to examine EPT infants, and the participants had a mean GA of 25.5 weeks, which was considerably lower than in previous studies. Nevertheless, the findings obtained from the whole-brain analysis are largely in agreement with previous studies in more mature cohorts (Anjari M et al. 2007; Rose SE et al. 2008; Anjari M et al. 2009; Lee AY et al. 2013) with, however, some differences. In contrast to Anjari et al (Anjari M et al. 2007). we found lower FA in the CR in the preterms compared with the term born controls. Rose *et al* (Rose SE et al. 2008) observed an FA increase in the splenium of the CC in their most immature subsample. Different findings between studies may be attributed to the participants' varying GA at birth, age at scanning, sample sizes, and differences in methodology.

In order to obtain more information about pathology underlying changes in FA, the diffusion tensor eigenvalues, representing diffusion along (AD) or perpendicular to the fiber tract (RD) were investigated (see online supporting information for Study I, Table S1). Reductions in FA may be due to a reduction in AD along the principal axis of the eigenvector λ_1 , or due to increases in RD in the two minor axes λ_2 and/or λ_3 . In white matter injury both myelin loss and axonal damage have been observed. With the help of animal models, specifically with the measurement of the averaged principal diffusivity in excised and fixed spinal cords from myelin-deficient rats (Gulani V et al. 2001) (Gulani V et al. 2001) and the quantification of the effect of dysmyelination on water directional diffusivities in brains of mice *in vivo*, characterized by incomplete myelin formation in the CNS (Song SK et al. 2002) an understanding has emerged that the eigenvalues of the diffusion tensor corresponding to the RD are more sensitive markers of myelination than FA. In our study we observed that in the EPT infants reduced FA was accompanied by a reduction in AD in the splenium and genu of the CC as well as the left ILF, and also in the frontal WM before correction for multiple comparisons. In the CR, reduced FA was accompanied by an increase in RD. Radial and axial diffusivity have been shown to be deranged in infants with diffuse WM changes on MR imaging and the authors concluded that the findings may reflect delayed myelination (Counsell SJ et al. 2006). The interpretation and biological significance of such findings is not straightforward and requires cautious interpretation. Animal studies have suggested that demyelination and alterations in the premyelination processes increase RD in the cerebral white matter with minimal effects on

AD (Gulani V et al. 2001). Axonal damage, on the other hand is thought to lead to decreased AD with a relatively smaller effect on RD in the two smaller axes of the diffusion tensor (Song SK et al. 2002). Another animal model study, investigating correlations between diffusional anisotropy and developmental changes in anatomy, with diffusion MRI and electron microscopy, showed that the axons progressed from tortuous to straighter and more parallel courses, forming orderly bundles as development proceeded. They conclude that, although myelination proceeds at the same time as these changes in axonal orientation, it is the change in fiber orientation rather than the development of myelin that is responsible for the evolution diffusional anisotropy (Takahashi M et al. 2000). Based on the above, our findings would imply either axonal damage in these regions, delayed myelination or changes in fiber organization. In fact, WM damage in preterm infants has been related to less mature fiber track development and organization (Cheong JL et al. 2009). However, one must keep in mind that our studies are focused on infants, born in the beginning of the third trimester where myelination has just began and that there is an abundance of premyelinating oligodendrocytes in the human cerebral WM. During this developmental phase, the early differentiating oligodendrocytes are beginning to ensheath cerebral WM axons, in preparation for myelination, which happens near term in the human (Kinney HC et al. 1988; Back SA et al. 2001; Back SA et al. 2002). Thus, we speculate that our findings are due to the presence of an immature brain and prematurity and not actual axonal damage in these infants. Nevertheless, an inaccurate placement of ROIs would lead to altered AD and RD values. However the placement of the ROIs was based on the TBSS results corrected for multiple comparisons, which were superimposed on the TBSS skeleton in the centers of the WM tracts; initially manually drawn on these regions and then automatically projected and placed on each subjects' individual FA map.

Reduced FA in the preterm brain in the splenium and genu of the CC was accompanied by an increase in MD and an increase in AD in our study. The growth rate of the CC has been investigated in the very preterm infant with cranial sonography, and the CC has been found to grow at a much lower rate postnatally than in utero (Anderson NG et al. 2005). Additionally, preterm infants with shorter CC (measured on cranial sonography) at TEA were shown to have a poorer neurodevelopmental outcome. Along the same lines, a conventional MRI study in very preterm infants has demonstrated smaller CC size (measured on true midsagittal MRI) in children who were born preterm (Rademaker KJ et al. 2004). A number of studies in children and adolescents have linked atypical CC size to impaired cognitive or motor outcome (Abernethy LJ et al. 2004; Nosarti C et al. 2004).

The asymmetrical findings, between brain hemispheres, regarding the relationship between FA and GA in the CR and ILF in WM is noteworthy. It has been shown in typical subjects that the right and left frontal lobes have different volumes as early as 20 weeks gestation, (Weinberger DR et al. 1982). However, the correlation with microstructural changes remains unclear.

In our study, EPT infants had significantly higher MD than the controls bilaterally in the EC, the genu and splenium of the CC, and the left ILF, (lower panel of Figure 3 in Paper I), which is in agreement with existing studies (Anjari M et al. 2007; Rose SE et al. 2008; Ball G et al. 2010). No regions were found where EPT infants had significantly lower MD than the controls.

At TEA, elevated MD values (Counsell SJ; MA Rutherford; et al. 2003) and reduced relative anisotropy values in WM (Huppi PS et al. 2001) have been associated with abnormal white matter assessed on MRI.

We found that fewer brain regions differed between the groups for MD than for FA, which, overall, is consistent with the findings by Lepomaki et al. (Lepomaki V et al. 2012). This might suggest that in various brain regions during different stages of WM development, FA and MD evolve with different rates of correlation. MD and FA represent two very different processes and, in fiber tracts without a high degree of organization, MD values may more closely reflect WM maturation than anisotropy values do (Schmithorst VJ et al. 2002). Decreases in MD parallel maturational processes of the brain, which include an increased concentration of macromolecules and cellular proliferation (Baratti C et al. 1999). FA appears to be related to myelination and maturational stage of oligodendrocytes but also to factors such as cellular membranes and cell density (Virta A et al. 1999). Alternatively, observed differences between preterms and term born controls in diffusion measures may be a result of altered packing and organization of premyelinated axonal fibers, increasing axonal thickness, alterations in axonal permeability and premyelination wrapping of the oligodendrocyte around the axon (Wimberger DM et al. 1995).

Region of interest analysis confirmed the findings from TBSS analyses for FA. For MD, the ROI analyses confirmed findings of significantly higher MD bilaterally in EC, genu of the CC and the left ILF (Table 2, Paper I).

It is essential to keep in mind that the interpretation of changes in the diffusion tensor is complex and should be performed with caution. Further complications in the interpretation of diffusion measure changes are due to the sensitivity of the diffusion tensor, and the anisotropy in particular, to a broad spectrum of other factors including image noise (Pierpaoli C and PJ Basser 1996; Basser PJ and S Pajevic 2000) artefacts, partial volume averaging between tissues in large voxels (Alexander AL et al. 2001), and underlying fibre architecture (for example, regions of crossing WM tracts).

4.2.3 Volumetric MRI findings (Paper III, IV)

4.2.3.1 Global volume data and correlation analyses

No significant differences between hyper- and normoglycemic groups were found with regards to global GM and WM volumes. Although infants with hyperglycemia had significantly smaller grey and white matter volumes than normoglycemic infants, these differences disappeared after controlling for CRIB score, sex and growth (z-scores for weight until TEA) (Table 4, Paper III). In the whole sample, there were significant negative correlations between hyperglycemic values in the assessed 24 hours, 48 hours, one-week periods and the GM and WM global volumes at TEA (Table 4, Paper III).

4.2.3.2 Voxel-based morphometry regional analyses

Hyperglycemic infants showed decreased regional grey and white volumes in several regions, and this remained after adjusting for sex and CRIB. The structural differences seen at TEA in the brains of hyperglycemic infants were more pronounced for glucose measurements obtained in the first 24 hours rather in the later periods. Infants who were hyperglycemic during this period had regions of decreased grey matter including the frontal lobe (orbito-frontal cortex, pre-frontal, and pre-central regions), parietal lobe

(post-central regions, precuneus), occipital lobe, temporal lobe (middle and superior gyri) and posterior insular cortex, bilaterally. The orbito-frontal cortex, pre-frontal region and left amygdala were also affected. White matter decreases were located adjacent to areas with grey matter decrease, especially in the anterior and posterior corpus callosum and cingulum. At TEA, infants exhibiting hyperglycemia in the first 48 hours after birth had areas of decreased grey and white matter volumes, bilaterally distributed, and predominantly in the temporal lobes, and the insula. The orbito-frontal cortex, pre-frontal region, and amygdala (bilaterally) were also affected. Correlations were seen between glucose measurement during the first week after birth and grey and white matter reductions in the frontal (orbito-frontal cortex, prefrontal), temporal lobes (inferior gyri bilaterally, left amygdala, parahippocampal cingulum), parietal lobe (post-central regions, precuneus), and occipital lobes. White matter decreases were seen in the temporal lobes (superior and inferior gyri bilaterally) anterior and posterior part of the cingulate cortex (Figure 4, Paper III).

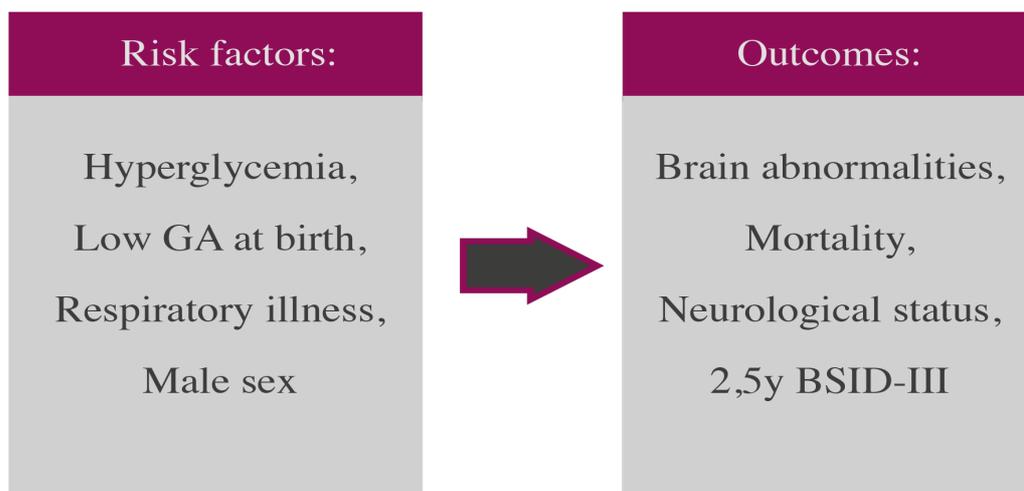
In the present study we did not find global brain volume group differences between hyper- and normoglycemic EPT infants. However, we did find that the exposure to hyperglycemia was associated with differences in both GM and WM regional volumes. Grey matter between-group differences occurred most prominently in the precuneus, temporal, occipital and insular cortices. The differences found in the precuneus are especially interesting. The precuneus region is known to have the highest levels of glycolysis in the brain in resting state conditions (Vaishnavi SN et al. 2010). A high rate of glycolytic activity due to systemic hyperglycemia could possibly make brain tissue more vulnerable to damage or dysfunction by enhancing a metabolism dependent cascade such as hyperglycemia-induced oxidative stress (Sharma R et al. 2010; Vincent AM et al. 2010). In agreement with previous studies in diabetic children with hyperglycemia (Perantie DC et al. 2007) the precuneus may have a specific vulnerability in hyperglycemic infants. This region belongs to the associative cortex with a central role in a wide spectrum of higher-order functions and complex cognitive processes (Cavanna AE and MR Trimble 2006). The precuneus also exhibits structural and functional connectivity with other cortical regions such as the occipital, motor, pre-frontal, temporal and insular cortices (Zhang S and CS Li 2012) which may also explain alterations in these regions. Of particular note is the involvement of the insular cortex; this region has been previously reported as affected in young children with glycemic dysregulation (Northam EA et al. 2009). This area plays a major role in control of emotions, working memory and selective visual-attention function (Dapretto M et al. 2006).

The group exhibiting hyperglycemia in the first week after birth had reductions in GM and WM volumes in the posterior cingulum, parahippocampal gyrus, amygdala, pre-frontal regions, motor cortex, insula bilaterally, and occipital regions, relative to the normoglycemic infants. An MR spectroscopy study in children with persistent hyperglycemia reported low metabolite ratios in the posterior parietal white matter, indicating possible dysfunction or reduced axonal density in this region (Sarac K et al. 2005). The decreased regional volumes found in our study may be due to compromised neurogenesis during development since hyperglycemia is associated with reduced insulin-growth factors, which are essential for neuronal growth, dendritic arborization and synaptogenesis (Bondy CA and CM Cheng 2004) (Ogilvy-Stuart AL and K Beardsall 2010). In adolescents with type 1 diabetes, a covariation between hyperglycemia, increased inflammatory markers and reduced insulin-like growth factor-1 levels has been previously shown (Van Sickle BJ et al. 2009; AboElAsrar MA et al. 2012).

In our study hyperglycemic infants were shown to have lower birth weight and weight at TEA than the normoglycemic infants, which might indicate poor growth in general. In addition, brain volumes between hyperglycemic and normoglycemic infants disappeared when results were adjusted for growth (z-scores for weight until TEA), also indicating that the impaired brain growth in the hyperglycemic infants may be a sign of a poor growth. In this context it is interesting to note that the insulin treatment during the first week after birth in very low birth weight infants led to reduced risk for hyperglycemia and ameliorated growth in parallel with increased insulin-like growth factor-I (IGF-I) bioactivity (Beardsall K et al. 2007). Furthermore, levels of IGF-1 were inversely correlated with risk for cognitive delay at 2 years, which was partly related to cerebellar volume (Ley D et al. 2013). Our findings of lower birth weight could be complementary to findings from a previous study investigating the association between early hyperglycemia and growth and development from hospital discharge to 2 years corrected age of very low birth weight infants (Ramel SE et al. 2013). They showed that the hyperglycemic infants had a slower rate of physical growth (weight, length, occipital-frontal head circumference) and that by 24 months, infants with more than 5 days of hyperglycemia were predicted to be 2 kilograms lighter than infants with no days of hyperglycemia. These findings are supported by findings from a study in EPT infants, where continuous intravenous infusion of recombinant human IGF-I and IGF binding protein 3 was shown effective in increasing serum concentrations of IGF-I and IGFBP-3 (Ley D et al. 2013).

4.3 RISK FACTORS AND OUTCOMES

One of the aims of this thesis work was to explore the respective contributions of immaturity at birth and in this population frequently present neonatal risk factors on outcome. Risk factors identified and investigated were early hyperglycemia, immaturity at birth (low GA at birth), respiratory illness (in terms of BPD, need for mechanical ventilation), and sex. Outcomes of interest were brain abnormalities, mortality, neurological status and neurodevelopment at 30 months corrected age.



4.3.1 Hyperglycemia and Mortality (Paper II)

By using receiver operating characteristic curves, we were able to test for the best diagnostic cutoff value to define hyperglycemia during the first 24 hours of life, for prediction of the risk for death in this particular population (Paper II). We tested these three cutoff levels for sensitivity and specificity, with death as the outcome, by using receiver operating characteristic curve analysis (Hanley JA and BJ McNeil 1982). The definition of hyperglycemia during the first 24 hours as a plasma glucose level of < 8.3 mmol/L was more sensitive and specific for death than were the other cutoff levels (7.6 or 10.0 mmol/L); therefore, that definition was used for coding of hyperglycemia.

The mean glucose values at 24 hours differed significantly between infants that survived and infants that died before TEA ($P=0.001$, Table 2, Paper II). The proportion of infants with hyperglycemia was significantly greater among infants who subsequently died (10 [53%] of 19 infants), compared with survivors (20 [21%] of 94 infants; $P=0.03$, Table 2, Paper II). Mortality rates did not differ in relation to the absolute number of days with hyperglycemic episodes during the first week of life for groups I to III. After adjustment for the unequal number of hyperglycemic episodes, an increase in hyperglycemic episodes showed weak evidence of a trend to increased mortality rates, from 6% for group IV to 16% for group V and 27% for group VI ($P=0.06$, by χ^2 test for trend). Multivariate logistic regression analysis revealed that hyperglycemia was a risk factor for death.

A few studies have shown associations between hyperglycemia and death and/or morbidity among preterm infants but in those studies, different glucose cutoff levels were used, different populations were studied, and different outcomes were analyzed. Most of those studies also concentrated on hyperglycemia during the first week of life. Kao et al examined the impact of hyperglycemia (blood glucose levels of ≥ 10 mmol/L) in preterm infants born at GAs of < 31 weeks and found increases in the rates of death and sepsis (Hall NJ et al. 2004) found longer hospital stays for infants born at GAs of < 29 weeks who were admitted with necrotizing enterocolitis and had blood glucose levels of ≥ 8.0 mmol/L. In a study of infants born at GAs of < 27 weeks, mortality rates were found to correlate with increasing glucose levels and repeated (≥ 4) incidents of blood glucose levels of ≥ 9.4 mmol/L (Hays SP et al. 2006; Heimann K et al. 2007) found that hyperglycemia (blood glucose levels of ≥ 8.3 mmol/L) in infants with birth weight of < 1000 g was associated with increased mortality rates and increased incidence of severe IVH. Our study could not demonstrate an association between hyperglycemia and sepsis or IVH but found associations of hyperglycemia during the first 24 hours after birth with death and with cerebral WM changes seen on MRI scans at term age.

4.3.2 Hyperglycemia, sex and Morbidities

In our study, boys spent longer time on CPAP compared to girls ($p=0.015$), but had similar rates of BPD. Three infants had cystic PVL on neonatal ultrasound and all were girls. Other neonatal variables and perinatal characteristics were similar for boys and girls (Study IV, Table 1).

4.4 BRAIN ABNORMALITIES (PAPER I, II, III, IV)

4.4.1 *Hyper- and hypoglycemia and Brain Abnormalities*

Multivariate logistic regression analysis revealed that hyperglycemia during the first 24 hours after birth was a risk factor for qualitatively defined WM reduction (Table 3, Paper II). No statistical association between hyperglycemia and gray matter abnormalities on structural MRI scans was observed (OR: 1.6 [95% CI: 0.13 to 18.2]; P = 0.7). No statistically significant association between hyperglycemia and sonographically assessed IVH was seen (OR: 1.4 [95% CI: 0.63–3.4]; P = 0.37). The results from logistic regression analyses indicated that hypoglycemia was not significantly associated with WMR (OR: 0.57 [95% CI: 0.22 - 1.47]; P = 0.24) after adjustment for sex and hyperglycemia (OR: 0.61 [95% CI: 0.22 - 1.68]; P = 0.34). WM reduction is illustrated in Paper II, Figure 3.

4.4.1.1 *MRI diffusion measures – comparison between hyper- and normoglycemic infants*

At TEA no significant differences in FA were found between infants exhibiting hyperglycemia in the first 24h and the normoglycemic ones (as seen in Paper III). However, infants exhibiting hyperglycemia during the first 48h after birth had lower FA, bilaterally in the level of the centrum semiovale, than the normoglycemic ones at TEA (figure 2, Paper III). These results remained significant after controlling for CRIB score and sex. No significant differences in FA were found between the hypoglycemic and normoglycemic infants for measurements taken in the 24, 48h or first week after birth.

The effect of hyperglycemia on brain abnormalities was further explored in Paper III with ROI analyses. No associations were found to the highest, or lowest glucose measurements or to the variation in glucose in the assessed 24 and 48-hour period. However, significant associations were found to the hyperglycemic load during the first 48 hours. Linear regression analyses found negative associations between FA measurements extracted from the ROIs in the right (model p=0.011) and left centrum semiovale (model p=0.25) and the hyperglycemic load (relative number of glucose episodes during the first 48h). After controlling for sex and CRIB score, only the multivariate model of FA in the right CS remained significantly negatively associated (model p=0.023) to lower FA measurements. A summary of these models can be found in Table 3, Paper III.

To the best of our knowledge this is the first study exploring WM microstructure and brain volumes in the EPT infant with early hyperglycemia. In agreement with our findings, a DTI study (Antenor-Dorsey JA et al. 2013) comparing term born youth with type 1 diabetes mellitus to normoglycemic controls demonstrated lower FA in the superior parietal lobule region. Altered FA may suggest that the region of the paracentral lobule and superior parietal lobule may be an area of particular susceptibility to hyperglycemia. White and gray matter microstructure could be affected by hyperglycemia through several mechanisms, including mitochondrial superoxide production (Brownlee M 2005).

Studies in mice have shown that hyperglycemia causes alterations in mitochondrial dynamics and function (Vincent AM et al. 2010) leading to accumulation of reactive oxygen species, oxidative stress, and impaired axonal transport in central nervous system axons, which can then result in axonal degeneration (Sharma R et al. 2010).

Alternatively, the FA alterations could also be due to other glycolysis-independent biological factors that are altered in parallel with hyperglycemic episodes. Despite the fact that FA correlations to early hyperglycemia did not reach significance on a corrected level, the uncorrected findings portray the same WM regions as those, of decreased WM, as shown in the volumetric analyses in the hyperglycemic infants (Figure 3, Paper III).

There were no differences in FA between the hypoglycemic and normoglycemic infants for the measurements taken in the 24, 48h or first week after birth. This is in opposition with previous research, which has identified MRI findings in the WM, deep nuclear gray matter, and cortical infarction as a result of hypoglycemia (Burns CM et al. 2008). The lack of findings in our study could be potentially explained by the knowledge gained from animal experiments (Brierley JB BA, Meldrum BS 1971a) studying the impact of hypoglycemia on the developing brain, which have suggested that in the newborn cerebral injury mediated by hypoglycemia is due to prolonged and severe, rather than transient or minor hypoglycemia in combination with mild hypoxia-ischemia. Hypoglycemia was seen in 26% (10/38) of the EPTs during the first 24 hours 34% (13/38) during the first 48h and 39% (15/38) during the first week after birth; however, due to tight glucose monitoring of the unstable infants and initiation of glucose infusion none of the preterm infants in our study exhibited persistent nor prolonged hypoglycemia.

4.4.1.2 Global volume data and Voxel-based morphometry analyses

Global brain volume group differences were not found, however, overall, hyperglycemic infants showed decreased regional grey and WM volumes comprising several regions after adjusting for sex and CRIB. The structural differences in the brains of hyperglycemic infants were more pronounced in the first 24 hours. Infants, hyperglycemic during this period had regions of decreased GM comprising the frontal lobe (orbito-frontal cortex, pre-frontal, and pre-central regions), parietal lobe (post-central regions, precuneus), occipital lobe, temporal lobe (middle and superior gyri) and posterior insular cortex, bilaterally. The orbito-frontal cortex, pre-frontal region and left amygdala were also affected. WM decreases were located adjacently to GM decreased with a major involvement of the anterior and posterior corpus callosum and cingulum.

At TEA, infants exhibiting, hyperglycemia in the first 48 hours of life presented areas of decreased grey and white matter volumes, bilaterally distributed, predominantly in the temporal lobes and insula. The orbito-frontal cortex, pre-frontal region, and amygdala were also affected. During the first week of life, grey and white matter reductions comprised the frontal (orbito-frontal cortex, prefrontal), temporal lobes (inferior gyri, amygdala, parahippocampal cingulum), parietal lobe (post-central regions, precuneus), and occipital lobes. White matter decreases were seen in the temporal lobes (superior and inferior gyri) anterior and posterior part of the cingulate cortex (Paper III, Figure 4).

Grey matter between-group differences occurred most prominently in the precuneus, temporal, occipital and insular cortices. In agreement with previous studies in diabetic children with hyperglycemia (Perantie DC et al. 2007) the precuneus showed a specific vulnerability in hyperglycemic infants. This is a region that belongs to the associative cortex with a central role in a wide spectrum of higher-order functions and complex cognitive processes (Cavanna AE and MR Trimble 2006). The precuneus also exhibit an increased structural and functional connectivity with other cortical regions such as the occipital, motor, pre-frontal, temporal and insular cortices (Zhang S and CS Li 2012) which may also explain alterations in these regions.

The decreased regional volumes found in our study may be due to compromised neurogenesis since hyperglycemia is associated with reduced insulin-growth factors, which are essential for neuronal growth, dendritic arborization and synaptogenesis (Bondy CA and CM Cheng 2004; Ogilvy-Stuart AL and K Beardsall 2010). In adolescents with type 1 diabetes, a covariation between hyperglycemia, increased inflammatory markers and reduced insulin-like growth factor-1 levels has been previously shown (Van Sickle BJ et al. 2009; AboElAsrar MA et al. 2012). Adjacent WM regions identified by the diffusion and volumetric analyses were located in the superior frontal and parietal cortex, the occipital cortex, corpus callosum and cingulum.

4.4.2 Respiratory Illness, Immaturity, Sex and Brain abnormalities

4.4.2.1 Respiratory Illness and Brain abnormalities

We aimed to investigate the respective contributions of respiratory illness and immaturity at birth to variation in FA and MD at TEA. Regression analyses in TBSS showed an association of BPD with lower FA in the splenium of the CC and the right ILF (Study I, Figure 4A, 4B), and this was confirmed by the ROI analyses, i.e. a diagnosis of BPD was associated with a -0.059 change (SE 0.023, 95% CI: -0.106 to -0.012, R2: 0.188) in FA in the splenium of the CC, and with a -0.045 change (SE 0.017, 95% CI: -0.080 to -0.011, R2: 0.202) in FA in the right ILF.

Regression analyses in TBSS demonstrated that preterms ventilated for more than two days had higher MD in the right EC (Figure 4C, Paper I) than preterms who were not ventilated at all or for fewer than two days. When GA was removed from the model, ventilation for longer than two days was still significantly associated with a 0.067×10^{-3} mm²/s (SE: 0.017, 95% CI: 0.034 to 0.101, R2: 0.232) change in MD. The summary statistics of these multivariate linear models are shown in Paper I, Table 3.

Respiratory morbidity correlated with FA in the splenium of the CC, right ILF and MD in the right EC, which is consistent with findings from other studies (Anjari M et al. 2009; Ball G et al. 2010; Shim SY et al. 2012). Despite using a similar image processing protocol, we could not replicate the findings of lower FA in the left EC and centrum semiovale in infants with BPD as demonstrated by Ball et al. 2010. The finding that respiratory morbidity is associated with WM microstructure is consistent with experimental data suggesting an association between respiratory disease and abnormal brain development (Chahboune H et al. 2009). It is uncertain why these specific WM regions should be affected, but it may be related to a vulnerable vascular architecture. The terminal branches of the posterior cerebral artery supplies blood to the splenium of the CC, whereas the ILF and EC are supplied by the terminal branches located between the deep and the superficial arterial systems of the middle and posterior cerebral artery, making these distal arterial territories equivalent to watershed zones and thus particularly susceptible to hypoxic ischaemic injury (Coley BD and MJ Hogan 1997). This suggests that this region of the brain may be particularly vulnerable either due to risks associated with acute lung disease or injury associated with patterns of ventilation.

4.4.2.2 Immaturity and Brain abnormalities

When comparing EPT infants with controls in TBSS and controlling for GA (Study I), no significant differences in FA or MD were seen, which suggests that GA at birth is a major contributor to variation in diffusion measures at TEA when a large GA range is examined. However, this appeared different when analyses were limited to the preterm infants only, where no significant associations between GA at birth and

diffusion measures were seen, indicating that within the range of EPT gestational ages, these diffusion measures were not affected in infants without focal lesions.

Regression models with GA at birth and respiratory morbidity variables as independent variables identified respiratory morbidity as a significant predictor of MD and FA in a number of WM tracts, whereas GA had a small effect for FA prediction, and no effect for prediction of MD. Similar findings were reported in a study in infants born <33 weeks GA (Bonifacio SL et al. 2010) that concluded that prematurity on its own was not a strong determinant of adverse brain development as assessed serially with diffusion imaging.

Nevertheless, the effect of increasing immaturity on WM microstructure has been used in DTI studies to characterize developmental changes in the preterm brain and to explain microstructural white matter developmental trajectories in preterm infants born at different gestational ages (Neil JJ et al. 1998; Miller SP et al. 2002; Partridge SC et al. 2004) These ROI based studies have shown regionally dependent anisotropy increases in white matter with increasing GA (Counsell SJ; JM Allsop; et al. 2003).

4.4.2.3 Sex and Brain abnormalities

In prospective studies, findings from DTI at TEA have been linked to later outcome (Krishnan ML et al. 2007). It has been shown that during the perinatal period male preterm infants are at increased risk of diffuse WM injury, which may be associated to learning and behavioral difficulties at school age (Bhutta AT et al. 2002; Foulder-Hughes LA and RW Cooke 2003). In the subsample of infants included in the whole brain TBSS analysis in our study, no differences were seen in diffusion measures between boys and girls (Paper IV), and no correlation between male sex and FA values at term age was found. Most likely, the lack of findings could be due to the fact that the subsample used in our TBSS analyses was not the optimal sample size to assure an adequate power to detect statistical significance.

In the subsample for which data for global and regional volume analyses were available, boys had overall larger brain volumes compared to girls although only statistically significant for the cerebellum ($p=0.029$). At voxel level, however, no significant sex differences were found. The present data support to some extent previous studies in preterm and term newborns (Vasileiadis GT et al. 2009; Knickmeyer RC et al. 2013) as well as in typical older children and adults (Groeschel S et al. 2010) demonstrating that males have larger brain volumes than females.

4.4.3 Sex and Neurodevelopmental outcome (Paper IV)

4.4.3.1 Neurological status

At age 30 months corrected, six of the 91 children fulfilled the criteria for CP (3 boys, 3 girls). Unspecific neurological signs were similarly distributed between boys and girls, and present in 12 boys and 14 girls. After excluding infants with moderate-severe WM abnormalities, abnormalities in the GM, or abnormalities in the cerebellum on T1-w and T2-w images ('exclusion group' $n=14$), two infants with CP remained in the sample (one boy, one girl), and no difference between boys and girls was seen in the frequency of unspecific neurological signs.

4.4.3.2 *BSID-III at 30 months corrected age*

Conventional structural MRI, sex and outcome

Boys had lower mean composite scores for the cognitive scale (94 ± 7 , mean \pm SD vs 98 ± 11 , $p=0.03$) and language scales (94 ± 13 vs 101 ± 15 , $p=0.04$) compared to girls (Table 2, Paper IV) whereas motor scores were not significantly different between the sexes. After the omission of data sets with focal lesions. Differences in the mean cognitive composite scores and the mean language composite scores remained between boys and girls with normal MRI on visual inspection (Table 2, Paper IV). When repeating the analyses excluding infants with delayed myelination and/or abnormalities in the cerebellum (findings persistent after the omission of the ‘exclusion group’, see above), the differences in the mean cognitive composite scores ($p=0.015$) and the mean language composite scores ($p=0.008$) between boys and girls persisted. Regression analyses confirmed that sex significantly influenced the variance in cognitive and language outcomes on the BSID-III scales, whereas delayed myelination, abnormalities in the cerebellum and number of days on CPAP did not. The BSID-III motor composite score was influenced by the number of days on CPAP but not by sex, delayed myelination or abnormalities in the cerebellum.

Brain morphology, sex and outcome

The subsample for which morphometric data were available was representative of the larger sample with regards to all perinatal characteristics and neonatal variables examined, as well as findings on conventional structural MRI. Consistent with the larger study sample, cognitive ($p=0.007$) and language ($p=0.026$) scores were poorer in boys than girls in this subsample, whereas the motor performance on the BSID-III and neurological status did not differ between the sexes.

Correlations between brain volumes and developmental scores differed between boys and girls on both global (Table 2, Paper IV) and regional levels (Figure 2A-C, Paper IV). In girls, cortical GM volume in pre-central regions (supplementary motor cortex and cingulate gyri bilaterally) correlated positively with BSID-III language composite scores (cluster size =423 voxels, $p<0.001$, Figure 2A). Supplementary motor cortex volumes also correlated positively with BSID-III fine motor scores (cluster=434, $p<0.001$, fFigure 2B, Paper IV). WM volumes in medial parietal areas (precuneus) correlated positively with BSID-III expressive language scores (cluster=136, $p<0.01$, figure 2C, Paper IV). In boys, no significant correlations between WM or GM volumes and BSID-III scores were found at regional level.

Our findings regarding the male disadvantage in terms of outcome are similar to other studies. At two years of age, the EPIPAGE study demonstrated a slightly lower developmental quotient in very preterm boys than in girls (Fily A et al. 2006). Male sex has previously been recognized as a risk factor for poor outcome after preterm birth. In the EPICure study, survival to discharge was lower, and frequency of neonatal problems was higher in EPT boys than girls. At age 30 months boys were more likely to have CP and impairment of cognitive function (Wood NS et al. 2000). Similarly, in the same cohort at age six years, cognitive, language, and educational difficulties were more pronounced in boys (Marlow N and H Budge 2005) in addition to persisting greater motor difficulties. In these studies, sex differences in brain imaging data were not investigated.

Sex differences in cognitive and language function could not be entirely explained by perinatal risk factors or brain morphology on conventional MRI in our study. It is well known that myelination is critical for normal neurodevelopment, that this process may be adversely affected by preterm birth, and that abnormal myelination often is associated with developmental delay (Volpe JJ 2008). Surprisingly though, delayed myelination on visual inspection of MR images did not contribute to the differences in outcomes between boys and girls. Whether these findings are related to compensatory, or “catch-up” mechanisms in boys between the time of imaging and the time of neurodevelopmental assessment, or if EPT boys truly have a higher constitutional risk for adverse outcome, as previously suggested (Hintz SR et al. 2006; Peacock JL et al. 2012), remains to be determined.

5 CONCLUSIONS

- i. WM microstructure, as reflected by MR diffusion measures, at TEA in EPT infants without focal brain lesions was significantly altered in several WM tracts compared to term born controls.
- ii. Within the EPT gestational age range, the degree of immaturity at birth was not independently associated with alterations of WM microstructure. Respiratory morbidity, on the other hand, was a major determinant of WM microstructure in this cohort.
- iii. The incidence of hyperglycemia and hypoglycemia in EPT infants during the first week after birth was 81% and 41% respectively.
- iv. Hyperglycemia on the first day after birth in EPT infants was associated with increased mortality rates and brain damage, as reflected by WM reduction at TEA.
- v. Early exposure to hyperglycemia was associated with alterations in WM microstructure and reduction in specific WM and GM volumes in EPT infants scanned at TEA.
- vi. EPT boys had poorer cognitive and language outcome at age 30 months compared to girls, whereas neurological and motor outcome was similar. Sex related differences were observed on neonatal MRI, including differences in the patterns of correlations between brain volumes and developmental scores at both global and regional levels.

6 GENERAL DISCUSSION

One major question in neonatology is whether the adverse outcomes in EPT infants are due to prematurity per se or to co-morbidities. Since these infants often suffer from a combination of morbidities that co-vary, it is difficult to disentangle causal relations between perinatal factors, perinatal brain injuries, and outcome. In an attempt to refine the preterm study population we therefore included only infants without evidence of focal brain lesions in studies I, III and IV, to rule out adverse effects on brain development due to lesions. The finding that there was no effect of immaturity on WM microstructure within the EPT range (23-26 weeks), but rather from respiratory disease, indicates that neonatal medicine should continue focusing on preventing these complications in order to prevent adverse neurodevelopment.

Our studies on hyperglycemia as a risk factor or early marker for mortality, WM damage, and impaired brain growth, are completely new in the field of neonatal medicine. In paper II it was evident that hyperglycemic infants were overall sicker than the normoglycemic infants and had a higher mortality, but that might not explain all of the variability in WM structure between the groups. When we adjusted for disease score in paper III, WM changes in certain brain regions of hyperglycemic infants remained and there were no differences in morbidities between the groups. We speculate that hyperglycemia may actually contribute to brain abnormalities in these infants and that studies to determine the effect of glucose control in extremely preterm infants might be considered.

It is well established that there is sexual dimorphism in brain structure in children and adults and that males have higher risks for neonatal morbidities but data on sex differences in early brain development are extremely limited. Our finding that EPT boys had poorer cognitive and language outcome at age 30 months compared to girls, although perinatal risks were similar, indicates that sex may be a separate biologic risk factor. The fact that in the female neonatal brain, but not in the male, volumes related well to outcomes, implies that there may be sex related growth differences. Future studies to address the sexual dimorphism in regional brain growth using neonatal atlases are ongoing.

As advances of perinatal health care promote the survival of more extremely preterm infants, the need to comprehend the possible effects of different factors on the brain, during their critical first days of life becomes essential, as it may have an impact on neurodevelopment later in life. This thesis aimed to elucidate some aspects of this complex matter with the ultimate goal to improve the outcomes of these infants

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