Markers and mechanisms of abnormal neurovascular development in the preterm infant

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Gothenburg 2023

Cover illustration: William Hellström

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ISBN 978-91-8069-107-9 (PRINT)
ISBN 978-91-8069-108-6 (PDF)

Printed in Borås, Sweden 2023
Printed by Stema Specialtryck AB

Dedicated to my grandfather Bo Jacobsson who was a

Pediatric Radiologist, and always encouraged me

Markers and mechanisms of abnormal neurovascular development in the preterm infant

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ABSTRACT

Background: The extremely preterm infant is at risk of lifelong neurodevelopmental impairments due to brain injuries or abnormal neurovascular development. Mechanisms are largely unknown and biomarkers for adverse outcomes are sparse. The growth factor insulin-like growth factor (IGF)-1 is a key regulator of neurovascular developmental processes and endogenous levels are low following preterm birth.

Aim of the thesis: To investigate the impact of growth factors on neurovascular development, e.g. retinopathy of prematurity (ROP), brain injury, brain volumes measured by magnetic resonance imaging, and neurodevelopmental outcome in preterm infants and in an animal model. In addition to identify possible biomarkers for abnormal neurovascular development in preterm infants.

Materials and Methods: *Paper I*: Associations between serum glucose levels, serum IGF-1, and ROP were explored in preterm infants (n=117) and in an oxygen-induced retinopathy/hyperglycemia mice model including IGF-1 substitution treatment. *Paper II*: Longitudinal serum Neurofilament Light (NfL, biomarker for axonal injury) levels were evaluated in preterm infants (n=221) as a biomarker for ROP, brain injury, and neurodevelopmental outcome at 2 years of age. *Paper III*: Longitudinal serum growth factor levels were correlated with total and regional brain volumes at term in extremely preterm infants (n=49). *Paper IV*: Longitudinal serum levels of NfL and IGF-1 and the association to neurodevelopmental outcomes at early school age were investigated (n=72).

Results: *Paper I*: Hyperglycemia was associated with lower IGF-1 levels, increased number of any ROP and with ROP severity. Hyperglycemia decreased endogenous IGF-1 expression, and IGF-1 treatment decreased ROP-associated vascular changes in the mice model. *Paper II*: NfL levels increased after birth and remained high, with increased levels independently associated with ROP development. High NfL levels were associated with unfavorable neurodevelopmental outcomes at 2 years. *Paper III*: Low serum IGF-1 levels

were independently associated with reduced total brain, white matter, cortical grey matter, deep grey matter, and cerebellar volumes. *Paper IV*: Unpublished results, see *Paper IV*.
Conclusion: IGF-1 may have a beneficial role in brain development and may have a protective role in ROP development. NfL may serve as a biomarker for ROP and adverse neurodevelopmental outcome.

Keywords: extremely preterm infant, brain development, brain volume, ROP, neurodevelopment, BSID, IGF-1, NfL

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SAMMANFATTNING PÅ SVENSKA

Barn som föds mycket för tidigt löper en ökad risk för akuta sjukdomar och skador som påverkar ögonen och hjärnan. Dessa tillstånd kan i sin tur leda till långsiktiga svårigheter för barnen. En mindre andel barn får svåra funktionsnedsättningar såsom cerebral pares (CP) eller intellektuell funktionsnedsättning, medan betydligt fler får mindre uttalade motoriska problem, inlärningssvårigheter eller synnedsättning. På senare tid har man också förstått att många för tidigt födda barn får autism eller ADHD (attention deficit hyperactivity disorder). Risken är störst för de barn som föds extremt för tidigt, dvs mer än tre månader före fullgången tid.

Den vanligaste och allvarligaste sjukdomen som drabbar ögonen hos för tidigt födda barn är ROP (retinopathy of prematurity). Den kännetecknas av en sjuklig tillväxt av blodkärlen i näthinnan och kan vid utebliven behandling ge en allvarlig synpåverkan. Många barn får också en påverkan på hjärnan, antingen i form av direkta skador (framförallt hjärnblödningar) men också på grund av en påverkad tillväxt och utveckling av hjärnan. Många kliniska riskfaktorer är gemensamma för ROP och hjärnpåverkan, exempelvis svår lungsjukdom eller bristande nutrition hos det nyfödda barnet. Det är därför sannolikt att det finns gemensamma mekanismer och också gemensamma markörer för sjukdom/skada och senare funktionsnedsättning. Trots omfattande forskning är skademekanismerna till stora delar okända och det finns idag inget enkelt och säkert sätt att tidigt identifiera riskbarn.

I denna avhandling studeras sambandet mellan tillväxtfaktorer i blodet, framförallt IGF (insulin-like growth factor)-1, och ROP, hjärnans utveckling och senare funktionspåverkan. IGF-1 har stor betydelse för hjärnans och ögats normala utveckling och man vet att för tidigt födda barn har lägre nivåer i blod än fullgångna barn. Våra studier visar att IGF-1 är lågt hos för tidigt födda barn med högt blodsocker och att dessa barn har en ökad risk för svår ROP. För att bekräfta sambandet mellan IGF-1 och ROP använde vi också en djurmodell och fann att risken för ögonsjukdomen minskade då möss fick behandling med IGF-1. Vi undersökte också IGF-1 i relation till hjärnans utveckling och fann att hela hjärnan, men också specifika områden i hjärnan, var volymmässigt mindre i fullgången tid hos barn som haft låga IGF-1 nivåer i blodet efter födelsen.

Vi undersökte också om så kallade hjärnskadeproteiner i blodet kunde fungera som en tidig markör för senare ögonsjukdom och hjärnpåverkan. Vi fann att markören NfL (neurofilament light), som stiger vid skador på nervceller, låg

högt hos för tidigt födda barn under veckorna efter födelsen. Barnen som hade de högsta nivåerna hade också en förhöjd risk för ROP och utvecklades sämre då de bedömdes vid 2 års ålder.

Vi har även undersökt kopplingen mellan nivåerna av IGF-1 och NfL tidigt efter födelsen med utvecklingen och neuropsykiatriska diagnoser i skolåldern. Dessa data är ännu inte publicerade, men återfinns i avhandlingen.

Våra studier tyder på att IGF-1 är viktigt för hjärnans utveckling och för att skydda mot ROP hos extremt för tidigt födda barn, men ytterligare studier krävs innan vi kan säga detta med säkerhet. NfL är en lovande tidig markör för ROP och avvikande utveckling, men man behöver undersöka vid vilken tidpunkt (ålder) prover skall tas och vilka gränsvärden som är kopplade till ökad risk.

LIST OF PAPERS

This thesis is based on the following papers, referred to in the text by their Roman numerals.

I. Bertan Cakir, **William Hellström,** Yohei Tomita, Zhongjie Fu, Raffael Liegl, Anna Winberg, Ingrid Hansen-Pupp, David Ley, Ann Hellström, Chatarina Löfqvist, Lois E. H. Smith. IGF-1, serum glucose, and retinopathy of prematurity in extremely preterm infants. *JCI Insight, 2020, October, Volume 5, Issue 19, e140363*

II. Ulrika Sjöbom*, **William Hellström*** Chatarina Löfqvist, Anders K. Nilsson, Ingrid Hansen-Pupp, David Ley, Kaj Blennow, Henrik Zetterberg, Karin Sävman, Ann Hellström**.** Analysis of brain injury biomarker Neurofilament Light and neurodevelopmental outcomes and Retinopathy of Prematurity among preterm infants. *JAMA Network Open, 2021, April, Volume 4, Issue 4, e214138*

*Shared first authorship

III. **William Hellström**, Lisa M. Hortensius, Chatarina Löfqvist, Gunnel Hellgren, Maria Luisa Tataranno, David Ley, Manon J.N.L. Benders, Ann Hellström, Isabella M. Björkman-Burtscher, Rolf A. Heckemann, Karin Sävman. Postnatal serum IGF-1 levels associate with brain volumes at term in extremely preterm infants. *Pediatric Research, 2022, June 9. Online ahead of print. PMID: 35681088*

IV. **William Hellström**, Chatarina Löfqvist, Ulrika Sjöbom, Anders K. Nilsson, Gunnel Hellgren, Liv Södermark, Staffan Nilsson, Matteo Bruschettini, David Ley, Henrik Zetterberg, Kaj Blennow, Ann Hellström, Karin Sävman. Neonatal serum levels of insulin-like growth factor (IGF)-1 and brain injury marker neurofilament light (NfL) are associated with autism at early school age in children born extremely preterm. *In manuscript*

RELATED PUBLICATIONS NOT INCLUDED IN THESIS

A. **William Hellström**, Ingrid Hansen-Pupp, Gunnel Hellgren, Eva Engström, Lennart Stigson, Karin Sävman, David Ley, Chatarina Löfqvist. C-Peptide suppression during insulin infusion in the extremely preterm infant is associated with insulin sensitivity. *Journal of Clinical Endocrinology and Metabolism (JCEM). Volume 104, Issue 9, 2019, pages 3902–3910*

B. Magnus Gram, Claes Ekström, Bo Holmqvist, Galen Carey, Xiaoyang Wang, Suvi Vallius Kvist, **William Hellström,** Niklas Ortenlöf, Alex Adusei Agyemang, Lois E. H. Smith, Ann Hellström, Alexandra Mangili, Norman Barton, David Ley. Insulin-like Growth Factor 1 in the preterm rabbit pup: characterization of cerebrovascular maturation following administration of recombinant human Insulin-like Growth Factor 1/Insulin-like Growth Factor 1-Binding Protein 3. *Developmental Neuroscience, Volume 43, 2021, pages 281-295*

C. Lisa M. Hortensius*, **William Hellström***, Karin Sävman, Rolf A. Heckemann, Isabella M. Björkman-Burtscher, Floris Groenendaal, Mats X. Andersson, Anders K. Nilsson, Maria Luisa Tataranno, Ruurd M. van Elburg, Ann Hellström, Manon J.N.L. Benders. Serum docosahexaenoic acid levels are associated with brain volumes in extremely preterm born infants. *Pediatric Research, Volume 90, 2021, pages 1177-1185*

*Shared first authorship

CONTENT

ABBREVIATIONS

ADHD	Attention deficit hyperactivity disorder
AUC	Area under the curve
BDNF	Brain-derived neurotrophic factor
BPD	Bronchopulmonary dysplasia
BSID	Bayley scales of infant and toddler development
CP	Cerebral palsy
cPVL	Cystic periventricular leukomalacia
DCD	Developmental coordination disorder
GA	Gestational age
GFAP	Glial fibrillary acidic protein
GMH	Germinal matrix hemorrhage
IGF-1	Insulin-like growth factor 1
IGFBP	Insulin-like growth factor binding protein
IQ	Intelligence quotient
IVH	Intraventricular hemorrhage
MABC	Movement assessment battery for children
MRI	Magnetic resonance imaging
NEC	Necrotizing enterocolitis
NfL	Neurofilament light
PDGF	Platelet-derived growth factor

PMA	Postmenstrual age
PND	Postnatal day
PVHI	Periventricular hemorrhagic infarction
PVL	Periventricular leukomalacia
RNA	Ribonucleic acid
OL	Oligodendrocyte
OR	Odds ratio
ROP	Retinopathy of prematurity
SD	Standard deviation
TEA	Term equivalent age
VEGF	Vascular endothelial growth factor
WMI	White matter injury
WPPSI	Wechsler preschool & primary scale of intelligence

DEFINITIONS IN SHORT

Extremely preterm birth	Birth before gestational week 28
Very preterm birth	Birth before gestational week 32
Term equivalent age	Postmenstrual age 40 weeks

THESIS AT A GLANCE				
	Paper I	*Paper II*	*Paper III*	*Paper IV*
AIMS	To investigate associations between serum glucose levels, serum IGF-1, and ROP in extremely preterm infants and in an experimental ROP/hyperglycemia model.	To evaluate longitudinal postnatal serum levels of NfL and GFAP in very preterm infants as possible biomarkers for ROP and neurodevelopmental outcome at 2 years.	To investigate links between longitudinal serum levels of growth factors IGF-1, BDNF, PDGF, and VEGF and brain volumes at term equivalent age in extremely preterm infants.	To investigate associations between early postnatal serum levels of IGF-1 and NfL and long-term neurodevelopmental outcome at early school age in extremely preterm infants.
METHODS	Clinical observational study, and experimental oxygen-induced retinopathy mice model of ROP. 117 infants, <28 weeks GA at The Queen Silvia Children's Hospital, Gothenburg and Skåne University Hospital, Lund. Included from 2005 to 2007, and 2013 to 2015.	Clinical observational study. 221 infants, <32 weeks GA at The Queen Silvia Children's Hospital, Gothenburg, Skåne University Hospital Lund, and Uppsala University Hospital, Uppsala. Included from 1999 to 2002, from 2005 to 2007, and from 2013 to 2015.	Clinical observational study. 49 infants <28 weeks GA at The Queen Silvia Children's Hospital, Gothenburg. Included 2013 to 2015.	Clinical observational study. 72 infants, <28 weeks GA at The Queen Silvia Children's Hospital, Gothenburg. Included 2013 to 2015.

THESIS AT A GLANCE				
	Paper I	*Paper II*	*Paper III*	*Paper IV*
RESULTS	Hyperglycemia was associated with decreased levels of IGF-1 and increased ROP severity. In the oxygen-induced retinopathy model, reduced insulin-signaling suppressed liver IGF-1 production and IGF-1 levels and increased neovascularization whereas exogenous IGF-1 improved retinal revascularization and decreased pathological neo-vascularization.	Serum levels of NfL increased following preterm birth and decreased 5-6 weeks postnatally. High levels of NfL during postnatal weeks 2-4 were independently associated with ROP and unfavorable neuro-developmental outcome at 2 years corrected age measured by Bayley scale of infant development.	High serum levels of IGF-1 during the first 4 postnatal weeks were independently positively associated with total brain volume, white matter volume, deep and cortical grey matter volume, and cerebellar volume at term equivalent age.	Unpublished results, see attached *Paper IV*.
CONCLUSIONS	IGF-1 might have a preventive role in the development of ROP.	NfL shows promise in predicting risk of ROP and adverse neurodevelop-mental outcome.	Higher levels of IGF-1 could be beneficial for early brain growth in the extremely preterm infant.	Unpublished conclusion, see attached *Paper IV*.

Abbreviations: BDNF: brain-derived neurotrophic factor, GA: gestational age, GFAP: glial fibrillary acidic protein, IGF-1: insulin-like growth factor 1, NfL: neurofilament light, PDGF: platelet-derived growth factor, ROP: retinopathy of prematurity, VEGF: vascular endothelial growth factor

1 INTRODUCTION

1.1 PRETERM BIRTH

Preterm birth is a major medical challenge in modern medicine. Annually, 15 million infants are born preterm[1]. Preterm birth is most commonly categorized by gestational age (GA) at birth, **Figure 1**. In Sweden, approximately 5.5% are born preterm, just below 1% are born *very* preterm, and *extremely* preterm infants account for 0.3% of all births, corresponding to 300-400 infants per year[2].

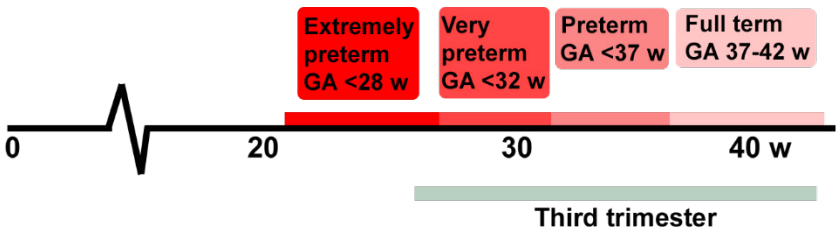

Figure 1. *Classification of preterm birth by gestational age at birth[3]. GA: gestational age, w: weeks*

1.1.1 SURVIVAL RATES

Preterm birth complications are the number one cause of death in infants below 5 years of age[4]. Over the last decades, the number of surviving infants born at low GAs has increased, leading to a growing population of immature infants[5]. The improved survival rate is the result of rapid advances in maternal and perinatal care, including the introduction of maternal prenatal injections of steroids for fetal lung maturation, surfactant treatment, and stricter guidelines regarding antibiotics, oxygen therapies, thermal care, and improved nutritional strategies[6-10]. Data on Swedish survival rates are shown in **Figure 2**.

According to recent data from the second Swedish Extremely Preterm Infants in Sweden Study (EXPRESS2), including all infants born before 27 weeks GA between 2014 and 2016, the one-year survival-rate was 77%[5]. In similar nationwide cohorts in Norway (Norwegian Extreme Prematurity Study-2 [NEPS-2]), France (Etude Epidémiologique sur les Petits Ages Gestationnels-

2 [EPIPAGE-2]) and UK/Ireland (EPICure 2), the one-year survival rates were 67%, 55%, and 51% respectively[11-13].

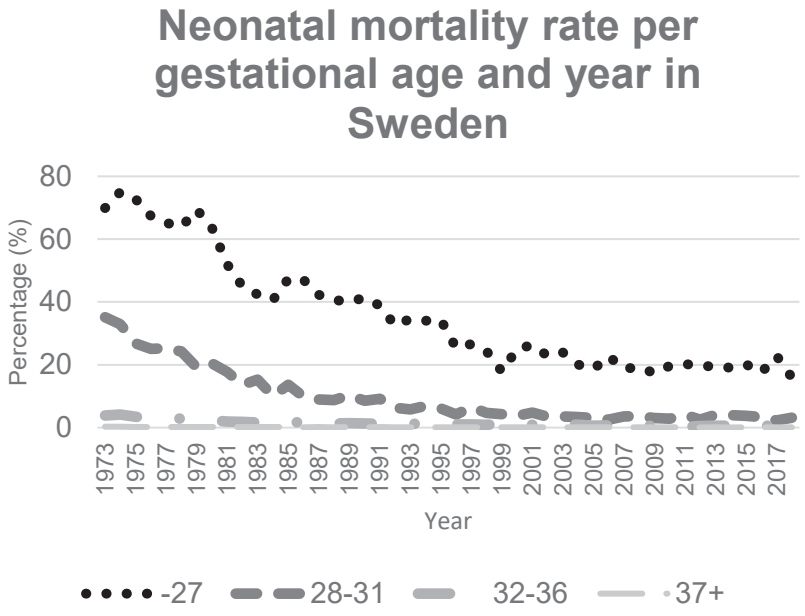

Figure 2. *Neonatal deaths (0–27 days), percentage of total births, by gestational age (weeks), 1973–2018, adapted from data from Socialstyrelsen[14].*

1.1.2 NEURODEVELOPMENTAL CHALLENGES IN PRETERM INFANTS

The prevalence of life-long neurodevelopmental disabilities remains high in preterm infants[15]. Several major organ systems are affected following preterm birth, however, the biggest concern for long-term sequelae is the brain. A recent Swedish cohort study, including 383 Swedish infants born before 24 weeks GA from 2007 to 2018, reported that 75% had at least one neurodevelopmental disorder[16].

Over the last decades, the general risk of severe macroscopic brain injuries such as severe intraventricular hemorrhage (IVH), periventricular hemorrhagic infarction (PVHI) and cystic periventricular leukomalacia (cPVL), with strong

associations with the most severe forms of sequelae such as cerebral palsy (CP) and intellectual disability has decreased in preterm infants[17,18]. Instead, a larger group of surviving preterm infants develop less pronounced neurological, cognitive, and behavioral disorders of a more subtle and complex nature. The underlying etiology is not fully known, nor is the proportion of what has historically been defined as structural brain injury and disruptions in the normal brain maturation as a result of preterm birth. Although the beneficial role of early detection and early intervention programs on long-term neurodevelopmental outcomes is well described[19], simple and reliable biomarkers to identify high-risk infants in the neonatal period are still lacking.

1.2 DEVELOPMENT OF THE BRAIN AND OF THE EYE

During the third trimester, the brain undergoes rapid development in the strictly regulated intrauterine environment, and several critical processes, including neuronal development and migration, synaptic development, and selective apoptosis, take place in a hierarchic fashion based on both cellular and genetic mechanisms **Figure 3**[20-23].

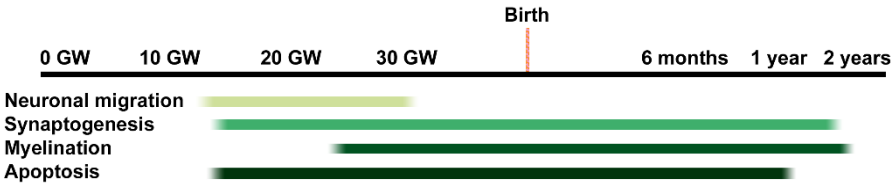

Figure 3. *Development of the human brain, schematic overview. GW: gestational weeks*

The third trimester is also the time of differentiation and proliferation of glial cells, including astrocytes, oligodendrocytes (OLs), microglial cells, Schwann cells, and ependymal cells. These cells have specific tasks and interact in a spatiotemporal scheme for optimal development which is specific to the human brain and sensitive to insult[24].

1.2.1 WHITE MATTER DEVELOPMENT

Myelination is initiated by OLs and is essential for effective signaling across the axon, and in providing axonal trophic support and protection[25,26]. The development and progression of the OL lineage cells occur in a strict order during brain development, and perinatal exposure to adverse events following preterm birth alters cellular maturation[27]. The OL lineage cells originate from the subventricular zone and radial glial progenitor cells, and the major cell type at 28 weeks GA is pre-OLs, which account for 90% of the cell population[28]. Myelination is initiated at approximately 30 weeks GA and is peaking during the first year of life[29-31]. Myelination develops regionally and is initiated centrally, starting in the visual system and expanding to the periphery in an occipital-frontal direction[32]. In the preterm infant, white matter dysmaturation and white matter injury (WMI) are proposed to be accompanied by grey matter disturbances, either by altered neurogenesis or disturbed neuronal maturation[33].

1.2.2 NEURONAL PROLIFERATION AND ORGANIZATION

Neuronal proliferation is initiated during early gestation and peaks at 2-4 months GA in the cerebrum and later in the cerebellum[34]. At 28 weeks GA, the specific position and organization of the cortical layers are microscopically visible[20]. The transient subplate is visible from 14-15 weeks GA and is most prominent before 35 weeks GA[35]. It is the initial cortical area getting thalamic sensory input, and is considered an area of crucial importance in several key processes in early cortical development and function[35]. It involves migrating cells and extrinsic axons which later will transform into white matter following myelination and it exceeds the cortical plate in thickness by 4 times at the beginning of the third trimester, only to diminish in size during the first 6 months of postnatal life[21]. Disruption of normal development in this zone following preterm birth and/or exposure to asphyxia and neonatal intensive care will severely impact sensory input[20]. It is suggested that subplate neurons are affected following periventricular leukomalacia (PVL)[36]. Further, the important process of neurogenesis is an ongoing event during the final trimester and is suppressed following preterm birth[37]. Most of the infants investigated in this thesis were born prior to the third trimester and thus exposed to altered physiological environment during this period.

1.2.3 CORTICAL EXPANSION

During the third trimester, the complex human cortex develops, and cortical folding is increased by 4 times[38]. In utero, the fetal brain increases in weight, from 80 grams in GA 22 weeks to around 400 grams at term equivalent age (TEA)[39]. During this more advanced stage of development, the superficial layers are, in a disproportional manner, increased in thickness[21]. The spatiotemporal dynamics of cortical development have been investigated thoroughly. Despite this, the underlying processes are still poorly understood.

1.2.4 RETINAL DEVELOPMENT

The development of the human eye via the embryonic diencephalon is initiated early in the first trimester. However, the development of the retina takes place both prenatally and postnatally[40]. The retina constitutes a part of the central nervous system sharing neuronal and vascular components with the brain. The underlying mechanisms of retinal vascularization are similar to those observed in cerebrovascular development[41]. The vascular development of the human eye is finalized in utero just prior to term birth. Angiogenesis in the maturing retina is facilitated by vascular endothelial growth factor (VEGF), expressed by neuroglia[41]. Several studies suggest that retinal neuro- and vascular morphology reflects cerebral microstructural integrity, brain injury, and dysmaturation[42-44].

1.3 NEUROVASCULAR INJURY AND DEVELOPMENTAL DISTURBANCES IN THE PRETERM INFANT

The preterm infant is at high risk of cerebral insults, however, the number of preterm infants with a macroscopic injury cannot explain the high prevalence of neurodevelopmental impairment in surviving preterm infants[45]. Current knowledge suggests an altered development of the brain in the preterm, also in infants without macrostructural brain injury[46,47]. While magnetic resonance imaging (MRI) at TEA and brain ultrasound during the neonatal period will identify macroscopic injuries, early and reliable biomarkers for abnormal neurovascular development and brain dysmaturation are lacking.

1.3.1 INTRAVENTRICULAR HEMHORRAGE

The most common macrostructural brain injury in preterm infants is IVH. Fragility of the highly vascularized capillary network in the germinal matrix and variability in cerebral blood flow are proposed as underlying mechanisms[48]. IVH diagnosed by cranial ultrasound was originally classified by Papile *et al.*, **Table 1**. However, in recent years, an updated classification was presented by Volpe *et al.*, primarily redefining Grade I as Germinal matrix hemorrhage and Grade IV as PVHI[34,49].

Table 1. *Original intraventricular hemorrhage (IVH) grading according to Papile et al.*[49].

Grade	Extension of hemorrhage
I	Subependymal hemorrhage
II	Intraventricular hemorrhage without ventricular distension
III	Intraventricular hemorrhage with ventricular distension
IV	Intraventricular hemorrhage with parenchymal hemorrhage

IVH occurs almost exclusively during the first postnatal week, and around 90% occurs during the first 3 days of life. IVH grade III and IV/PVHI remain severe injuries affecting approximately 10% of extremely preterm infants with persisting neurodevelopmental impairment, most importantly CP, in as many as 50-75%[50-52]. In relation to GA, severe IVH/PVHI affects approximately 21%, 8%, and 2% of infants born before 25, 27, and 31 weeks GA, respectively[53]. Recent studies also suggest that low-grade IVH (grade I and II), are associated with reduced cortical volume, reduced blood flow in grey matter, as well as slightly increased risk of CP[54-57].

1.3.2 WHITE MATTER INJURY AND DYSMATURATION

The dominant pathology underlying neurodevelopmental disorders in preterm infants is WMI with affected oligodendroglia, accompanied by impaired axonal development. More subtle alterations in white matter development following preterm birth are generally referred to as either diffuse white matter damage or WMI. It may, however, be more suitable to characterize it as

abnormal or arrested maturation, reflected by an impaired organization and reduced volumes of white matter. Studies have reported some degree of WMI in up to 50% of infants born preterm, however, the interstudy variability is high[58,59]. The previously not-so-uncommon macroscopic focal PVL is now only rarely observed. Instead, a more common form of non-cystic or diffuse WMI is seen in infants born very and extremely preterm.

1.3.2.1 PATHOPHYSIOLOGY AND CELLULAR MECHANISMS

The main pathogenetic mechanism in diffuse WMI is an affected oligodendroglia cell lineage and, more specifically, a decrease in pre-OLs, **Figure 4**. This results in an increase of oligodendroglia progenitors but these cells do not have the capacity for complete development and are unable to complete differentiation to mature myelinating cells. The disruption of normal pre-OLs maturation and failure of differentiation results in hypomyelination[60]. In addition, OL dysmaturation is accompanied by damage to immature axons[61,62]. The axonal injury is characterized by the disintegration of axons and neurons in the cerebral cortex, cerebellum, and basal ganglia[24].

Pathogenic factors implicated in diffuse brain injury and dysmaturation include ischemia and inflammation. Activation of immune responses during critical neurodevelopmental phases has lasting neurological and neurocognitive effects[63,64]. Potential targets for inflammation, other than oligodendroglial development, include endogenous stem cells, neuronal migration and survival, synaptogenesis, as well as epigenetic changes[65].

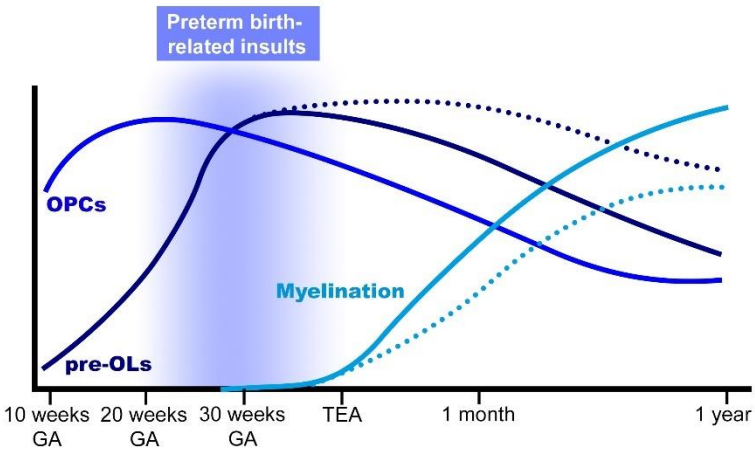

Figure 4. *Schematic overview of fetal and perinatal development of OPCs, pre-OLs, and brain myelination. The population of pre-oligodendrocytes peaks in the third trimester (dark blue line), while the oligodendrocyte precursor cells (blue line) peak earlier in gestation. The initiation of myelination occurs prenatally and is accelerated following birth (light blue line). Following preterm birth, infants are heavily exposed to perinatal insults during the most active stage of pre-oligodendrocyte proliferation, leading to impaired differentiation to myelin-producing oligodendrocytes. This results in the increased number of pre-oligodendrocytes and impaired myelination, which characterizes WMI in the preterm infant (dotted dark blue and dotted light blue line). Figure adapted from original figure by Van Tilborg et al. 2018[27], figure data originating from Back et al., 2001[28] and Buser et al., 2012[60]. OPC: Oligodendrocyte progenitor cell, OL: oligodendrocyte*

1.3.3 PRETERM BRAIN VOLUMES

Very and extremely preterm infants have disturbed brain growth, and brain maturation is delayed. The underlying cause is likely multifactorial and is suggested to include both primary brain *injury* and a secondary *disturbance* in genetically programmed brain maturation[66-69].

At TEA, global brain volumes are generally reduced in preterm infants[70-72], and the volume reduction is more prominent in infants with lower GA at birth[71-74]. Several studies have shown alterations in cortical grey matter, basal ganglia, cerebral white matter, and corpus callosum size in preterm infants compared with term controls[24,75,76]. This pattern seems to persist during childhood. Ment

et al. reported disturbed cerebral maturation between the ages of 8 to 12 years in preterm infants compared to term born controls, with both less white matter gain and grey matter reduction over time[77]. Further, in a recent meta-analysis by Schmitz-Koep B *et al.*, including data from 538 preterm infants and investigating brain volumes from 1.1 to 28.5 years of age, the cerebral grey matter was continuously reduced in preterm infants up to early adulthood and white matter volumes were notably low in adolescence in preterm teenagers compared to term controls[78]. However, some studies have also reported larger regional volumes, primarily in the frontal and parieto-temporal cortex in the preterm infant compared to term controls and in brain regions involved in visual processing in the extremely preterm infant[70,73,79-81].

1.3.3.1 FACTORS LINKED TO VOLUMETRIC BRAIN ALTERATIONS IN PRETERM INFANTS

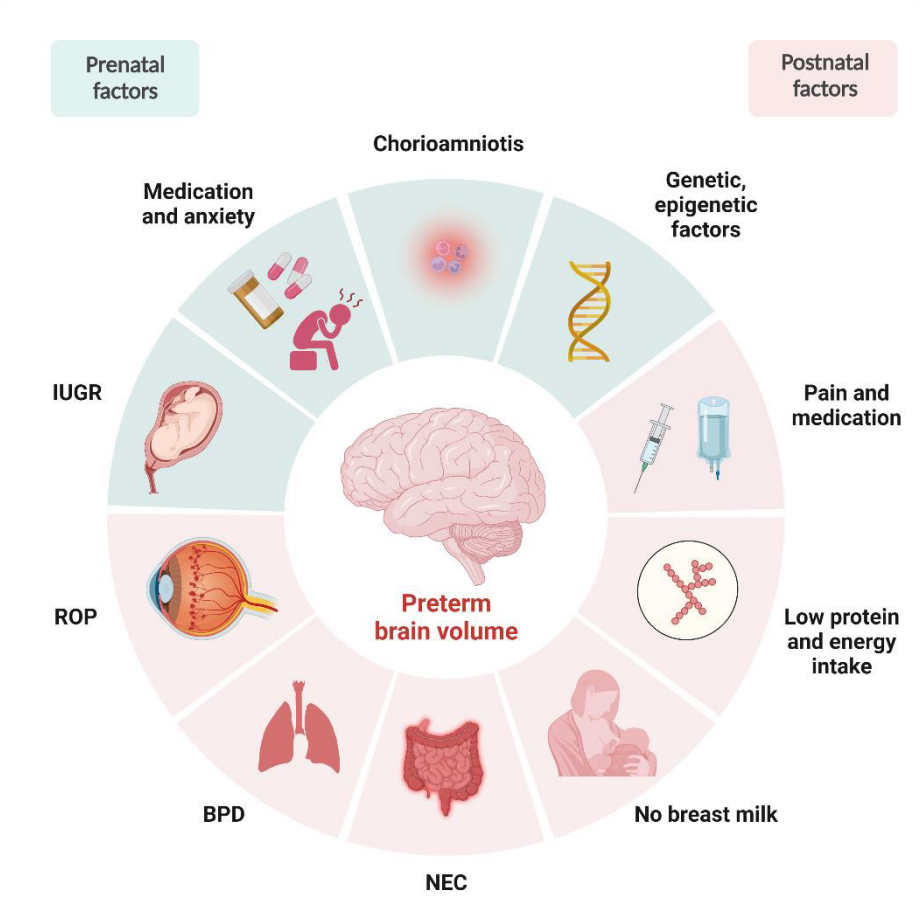

Figure 5. *Risk factors for altered brain volume in preterm infants based on clinical associations. BPD: bronchopulmonary dysplasia, IUGR: intrauterine growth restriction, NEC: necrotizing enterocolitis, ROP: retinopathy of prematurity. Created with BioRender.com.*

Several clinical risk factors may influence brain morphology around TEA in preterm infants, **Figure 5**. A more comprehensive list of perinatal risk factors associated with brain morphology at TEA is provided on the following pages (summary based on review by Boardman *et al.*[82]), **Table 2**.

Table 2. *Factors associated with brain morphology alterations in the preterm infant.*

Variable	Population	Result	Author
Maternal anxiety	251 infants ≤ 32 weeks gestational age (GA)	Increased stressful maternal events were associated with higher axial, radial, and mean diffusivity in the left uncinate fasciculus as well as higher axial diffusivity in the right uncinate fasciculus at term equivalent age (TEA)	Lautarescu *et al.* 2020[83]
Maternal antide-pressants	177 infants < 32 weeks GA	At magnetic resonance imaging (MRI) performed at 32 and 40 weeks, maternal SSRI exposure was associated with increased fractional anisotropy (FA), decreased measures of diffusivity in superior white matter, and decreased FA in basal ganglia and thalamus	Podrebarac *et al.* 2017[84]
Chorio-amniotis	90 infants ≤ 32 weeks GA	Histological chorioamnionitis associated with lower FA in several cerebral anatomic regions including in the inferior longitudinal fasciculi, cingulum cingulate gyri, centrum semiovale, genu and limbs of the internal and external capsule, and cerebellum at TEA	Anblagan *et al.* 2016[85]
Intra uterine growth restriction (IUGR)	28 preterm infants	IUGR infants had reduced intracranial volume (ICV) and cortical grey matter at early postnatal MRI and at TEA	Tolsa *et al.* 2004[86]

Variable	Population	Result	Author
Genetic/ Epi-genetic	83 preterm infants	Two genetic variants were associated with white matter abnormality	Boardman *et al.* 2014[87]
	72 infants <33 weeks GA	Associations between fatty acid pathways and variability in cerebral white matter development	Krishnan *et al.* 2016[88]
	194 infants <33 weeks GA	Polygenic risk scores for neuropsychiatric disease associated with lentiform volume at TEA.	Cullen *et al.* 2019[89]
Pain	155 infants ≤32 weeks GA	Early and late skin breaks. Large number of early skin-breaks were associated with less thalamic volume increase at MRI at 32 and 40 weeks post menstrual age as well as variations in thalamocortical pathways	Duerden *et al.* 2018[90]
	51 infants <32 weeks GA	Invasive procedures associated with decreased total brain, basal ganglia, and thalamus volume as well as decreased functional connectivity	Schneider *et al.* 2018[91]
Medi-cations	138 infants ≤32 weeks GA	MRI performed at 32 and 40 weeks showed that Midazolam intake was associated with smaller hippocampus and increased mean diffusivity	Duerden *et al.* 2016[92]
	58 infants <28 weeks GA	Higher morphine exposure before TEA associated with lower total brain and cerebellar volume at TEA MRI	Tataranno et al. 2020[93]

Variable	Population	Result	Author
Protein and energy intake	49 infants <30 weeks GA	High energy intake and lipid intake the first 2 postnatal weeks associated with increased total brain and basal nuclei volume, as well as FA in selected WM tracts	Schneider *et al.* 2018[94]
	131 infants <31 weeks GA	Cumulative fat and enteral intakes in the first 3 weeks of life were linked to increased cerebellar, basal ganglia and thalami volumes. Cumulative enteral, caloric, and fat intake were linked to FA in the posterior limb of the internal capsule	Coviello *et al.* 2018[95]
	42 infants ≤ 30 weeks GA	Increased lipid and energy intake the first 2 postnatal weeks associated with improved MRI scores at TEA	Beauport *et al.* 2017[96]
Breast milk intake	68 infants < 32 weeks GA	Infants receiving breast milk had increased total brain volume, cerebellar, and amygdala-hippocampus volumes, and improved microstructural organization in the cerebellum, corpus callosum, and posterior limb of capsula interna, as compared to infants receiving formula	Ottolini *et al.* 2020[97]

Variable	Population	Result	Author
Retino-pathy of prematur-ity (ROP)	52 infants <31 weeks GA	ROP (any grade) associated with reduced total brain, reduced unmyelinated white matter and cerebellar volume	Sveinsdó-ttir *et al.* 2018[98]
	98 infants <28 weeks GA	Severe ROP associated with lower FA in posterior WM and lower regional volumes	Glass *et al.* 2017[99]
Lung morbidity	119 infants <32 weeks with birth weight <1500 g + 21 term-born infants	The severity of respiratory illness/days on ventilator associated with deep nuclear grey matter (GM) volume/relative ICV and nuclear GM volume	Inder *et al.* 2005[71]
	93 preterm infants	Infants with chronic lung disease had increased radial diffusivity as well as decreased FA in the centrum semiovale, corpus callosum, as well as the inferior longitudinal fasciculus	Ball *et al.* 2010[100]
Necrotiz-ing entero-colitis (NEC)	155 infants <30 weeks GA	NEC with sepsis was associated with decreased diameter of the cerebellum and increased unilateral ventricular diameter	Lee *et al.* 2014[101]
	192 infants <30 weeks GA	NEC/sepsis was associated with white matter abnormality at TEA	Shah *et al.* 2008[102]
	33 infants <32 weeks GA	Surgically treated NEC was associated with more severe white matter injury at TEA than spontaneous intestinal perforation surgery	Shin *et al.* 2016 [103]

1.3.3.2 THE CONSEQUENCES OF ALTERED BRAIN VOLUMES - ASSOCIATIONS WITH NEURODEVELOPMENT OUTCOME

Reduced brain volumes in preterm infants at TEA are associated with neurodevelopmental outcome at 2 and 5 years of age, including motor, neurosensory, cognitive, and behavioral impairments[104-107]. Both associations between global cerebral volumes and regional brain volumes and neurodevelopmental outcomes have been investigated. In volumetric studies in the preterm infant, the brain is commonly subdivided into white matter and cortical and deep grey matter. In addition, the cerebellum, hippocampus, and corpus callosum are frequently targeted in analyses. As summarized by Kieviet *et al.*[108], reductions in total brain volume, white and grey matter, cerebellar volume, corpus callosum, and hippocampus volumes are linked to lower intelligence quotient in children born very preterm[109-117]. Further, smaller total brain volumes were associated with impaired executive functions[116]. Reduced white matter volumes are linked to reduced language, memory, and executive functions[116,117] and grey matter volume to memory[116]. Cerebellar volumes are associated with memory, motor skills, and executive functions[116], and corpus callosum with language, memory, motor skills, and executive functions[112,116,118,119].

1.3.4 RETINOPATHY OF PREMATURITY

The preterm infant is at risk of impaired neurovascular development, resulting in the retinal disease retinopathy of prematurity (ROP). ROP is a major reason for loss of vision in children, and annually approximately 20,000 children become blind or severely visually disabled as a result of ROP[120]. The incidence of ROP varies globally due to heterogeneity in critical care regimes and in regional survival. In Sweden, approximately 32% of infants born <31 weeks GA develop any form of ROP, and in preterm infants born <27 weeks GA, 20% require treatment[121,122].

Schematically, ROP is a two-phased disease, with an initial phase of vascular arrest, due to hyperoxia which downregulates VEGF, followed by a second phase of uncontrolled vessel growth/neovascularization orchestrated by growth factors including insulin-like growth factor (IGF)-1, and VEGF, as

illustrated in **Figure 6**[123]. Neovascularization could lead to detachment of the retina if left untreated[123].

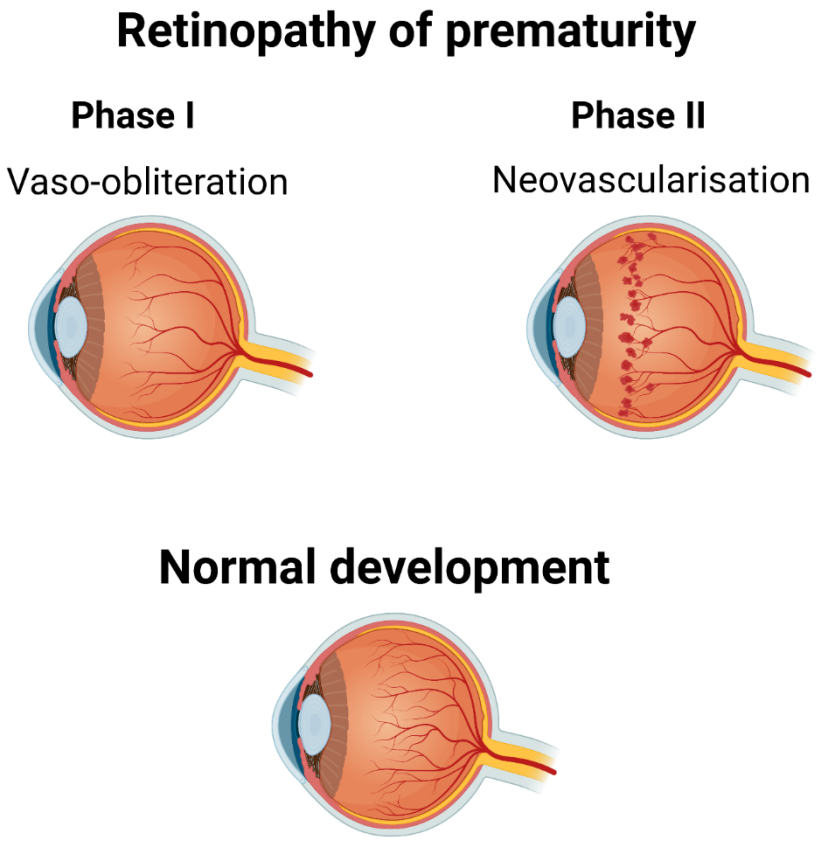

Figure 6. *Retinopathy of prematurity, with a bi-phasic progression involving vaso-obliteration, followed by neovascularization. Neovascularization could result in retinal detachment and blindness if untreated. Created with BioRender.com.*

Many risk factors have been associated with ROP, such as oxygen exposure (concentration, variability and duration), immaturity, low birth weight, and prolonged mechanical ventilation[124]. However, a wide range of other, both maternal and perinatal factors, such as insufficient nutrition, insulin treatment, sepsis, thrombocytopenia, anemia, blood transfusions, and elevated serum

glucose levels are also associated with ROP[124]. Treatment regimes for more severe forms of ROP now include laser therapy and anti-VEGF therapy[125], the latter being easier to administer intravitreally but with unknown long-term systemic effects.

1.3.4.1 RETINOPATHY OF PREMATURITY AND NEURODEVELOPMENT

Although the mechanisms are not fully understood, severe ROP has commonly been associated with later neurodevelopmental impairment[126,127] and reduced brain volumes[98]. Recent results indicate an impact of preterm birth on microstructures of the central nervous system[128-130]. As previously mentioned, recent research has emphasized the possible role of the retina as a proxy for cerebral integrity[131]. Clinical studies in adults have suggested a link between brain abnormalities such as MRI-characterized deep white matter hyperintensities, microbleeds, and neurodegenerative morbidities and the structural integrity and complexity of the retinal vasculature networks, commonly measured and quantified by fractal dimensions[44,132,133]. The association between ROP and neurodevelopmental outcome as well as common risk factors for ROP and impaired brain development, suggest that there may be common mechanisms and possibly common biomarkers for neurovascular injury of the eye and brain.

1.4 DIAGNOSIS OF BRAIN INJURY AND ABNORMAL MATURATION

Imaging techniques have improved enormously in recent years and now provide robust bedside examinations as well as technically sophisticated diagnostic options to detect subtle abnormalities.

1.4.1 CRANIAL ULTRASOUND

Cranial ultrasound has been a cornerstone for early detection of cerebral injuries and malformations in newborn infants for almost 50 years. It enables rapid, non-invasive, bedside detection and monitoring of intraventricular hemorrhages but also of macroscopic white matter abnormalities, including cysts and infarctions as well as ventricular dilatation, which are all linked to neurodevelopmental disorders. Although cranial ultrasound is an excellent tool

in detecting and monitoring macroscopic injuries and allows repeated examination in very immature infants without radiation, it is currently inferior to MRI in detecting subtle brain anomalies, e.g., white matter abnormalities.

1.4.2 MAGNETIC RESONANCE IMAGING

The addition of MRI and, more recently, advanced post-processing techniques, have provided valuable new insights into preterm brain development. Various approaches to quantify brain development and injury in the preterm include volumetric MRI, diffusion weighted MRI with diffusor tensor imaging and measures including fractional anisotropy, mean, axial and radial diffusitivity, proton MR spectroscopy, and resting-state functional MRI, and the predictive ability for assessing long-term neurocognitive outcomes seems very promising[134]. The signal intensity of neonatal, predominantly unmyelinated WM, has been specifically addressed and developed in recent years[135,136].

1.5 LONG-TERM NEURODEVELOPMENTAL OUTCOMES IN THE PRETERM

1.5.1 COGNITIVE OUTCOME

While the risk of severe motor injury is high following extremely preterm birth, the risk of suboptimal cognitive development is even higher, with approximately a third of extremely preterm infants classified as having intellectual disability at early school age[147]. A meta-review including 3,500 preterm infants showed, on average, a 12-point lower intelligence quotient (IQ) score compared to those born at term, and a very tight relationship between GA at birth and cognitive outcome[137], but IQ is also affected by other factors such as sex and maternal education[138]. Studies have also repeatedly shown that early low IQ persists into adulthood, with relatively small variability[139]. Further, very preterm infants or very low birth weight infants have impaired executive functions, and attention, also when adjusted for IQ[140-143]. In addition to being at increased risk of global cognitive impairment, many infants have speech disorders affecting language processing, short-term phonological memory, and articulation[144].

1.5.1.1 MAJOR COGNITIVE IMPAIRMENT

Intellectual disability is an important cognitive impairment among those born preterm, and is defined by an IQ below 70 (-2 standard deviations [SD]) with impaired adaptive skills[145]. The prevalence of intellectual disability among very and extremely preterm infants is high compared to the normal population where approximately 0.9% develop intellectual disability[146]. A Swedish study showed an odds ratio (OR) of 14.5 when born at 24 weeks GA compared with term born infants, with decreasing risk with increasing GA at birth[146]. According to the Swedish EXPRESS-study, including infants born <27 weeks GA, the prevalence of moderate to severe cognitive impairment (<-2SD) was 11% at 2.5 years of age and increased to 30% at early school age[147,148].

1.5.1.2 MINOR COGNITIVE IMPAIRMENT

While intellectual disability is common in extremely preterm infants, an even larger group develops learning difficulties and sub-optimal IQ, generally defined as IQ 70-85. In the EPICure study, extremely preterm infants scored lower than peers for cognition, reading, and mathematics, with a 10-fold increase in the need for special educational support at the age of 11 years[149]. In the Swedish EXPRESS study, the prevalence of mild cognitive impairment was 24% at 2.5 years of age and 30% at early school age[147,148].

1.5.2 MOTOR OUTCOME

1.5.2.1 MAJOR MOTOR IMPAIRMENT – CEREBRAL PALSY

CP is characterized by a non-progressive disruption to the motor center of the developing brain[150]. In extremely preterm infants, CP prevalence range from 7-20%, and the risk of developing CP is inversely related to GA at birth[151,152]. CP is strongly linked to macroscopic brain injuries, such as severe IVH/PVHI but most importantly marked WMI[153] with an OR for CP at 5 for severe IVH/PVHI and 15 for WMI[154]. CP is also linked to a wide range of underlying intrauterine pathologies, such as fetal growth restriction, placental vascular pathologies, infections in utero, and inflammation[155]. CP is heterogenous with a large variability in motor function. It is functionally classified according to the Gross Motor Function Classification System, a 5-level system based on individual mobility. CP is strongly associated with other neurodevelopmental disorders such as cognitive impairment, poor vision and neuropsychiatric disorders. Novak *et al.* performed a systemic review in 2012 and concluded that approximately 1 out of 2 children with CP had an intellectual disability, 1

out of 3 lacked the ability to walk, and 1 out of 4 lacked speech[156]. In the EXPRESS study, 76% had at least one additional neurodevelopmental impairment at 6.5 years of age[157] and approximately 45% of children with CP develop autism or attention deficit hyperactivity disorder (ADHD), with an association to WMI[158].

1.5.2.2 MINOR MOTOR IMPAIRMENT

Although only a small fraction of infants born preterm are affected by CP, a much larger group have less pronounced motor difficulties. The most common mild motor difficulty in the preterm population is developmental coordination disorder (DCD). DCD involves impaired motor function that interferes with daily life that cannot be explained by CP or other impairments[159].

In the Swedish EXPRESS study, 37% had DCD at early school age, with clear links to cognitive and behavioral disturbances[160]. Internationally, the prevalence within the preterm community usually varies between 10-50% depending on classification[161-164]. In children born term, the corresponding number is 5-6% at early school age[165]. DCD is associated with increased risk of cognitive deficits and neuropsychiatric disorders in particular[166-168]. Preterm infants diagnosed with DCD experience lasting effects throughout childhood[169].

1.5.3 NEUROSENSORY IMPAIRMENTS IN THE PRETERM INFANT

According to the EXPRESS study, 8.8% of children born before 27 weeks GA had severe visual impairment, and 2.1% were blind at early school age[170]. In addition, almost 38% of had some major ophthalmologic abnormality, including strabismus and refractive errors, compared to 6.2% in term-born children. The risk of visual and eye impairments is strongly linked to GA and can, to a certain degree, be the result of severe ROP requiring treatment[171,172]. Furthermore, preterm infants are at higher risk of impairment of more complex tasks involving the visual system, such as visual perception and motor integration, which are also associated with cognitive function, fine motor development, and academic performance in school[173,174]. These problems are generally defined as cerebral visual impairment, i.e. visual problems resulting from pathology of the brain rather than the eye[175,176].

A relatively small number of preterm infants develop hearing impairments, although the risk is increased compared to term infants. In Sweden, less than

1% had severe hearing disorders at 2.5 years of age, and 2% (0.5% severe) had moderate to severe hearing disorders at early school age[147,148].

1.5.4 NEUROPSYCHIATRIC DISORDERS

In recent years, neuropsychiatric disorders including autism spectrum disorders (ASD) and ADHD have been highlighted as major long-term challenges in preterm infants. In the first report from the EPICure study, 23% of extremely preterm infants at the age of 11 fulfilled the criteria for at least one neuropsychiatric disorder, with high prevalence of ASD and ADHD of the inattentive subtype[177,178].

1.5.4.1 AUTISM SPECTRUM DISORDERS

Following the EPICure study, several other studies have reported an odds ratio of up to 10 for developing ASD following preterm birth[179-181]. A Swedish population-based study of infants <28 weeks GA reports 6% autism between 1973 and 2013, and a recent study of infants <24 weeks GA reports 24% ASD, while ASD is found in approximately 1% of the general population with a strong genetic component[16,182,183]. The preterm population also differs from the general population regarding specific risk factors, such as low GA, abnormal placenta findings, and exposure to inflammation[16,180,181,184,185]. The link to neuroinflammation is highlighted by the increased risk for autism following chorioamnionitis in the preterm group[186]. In the experimental setting, neuroinflammation in the immature brain results in autism-like behavior[187]. All together, external factors that affect brain development are also associated with ASD specifically in the preterm group[179,188].

1.5.4.2 ATTENTION DEFICIT HYPERACTIVITY DISORDER

Preterm infants have increased risk of ADHD symptoms and the risk is most pronounced for the most immature infants with an OR of 3 compared with term infants[189]. The EPICure study reported that 11% of children born <26 weeks GA fulfilled criteria for ADHD, with a predominance for the inattention subtype[177]. In a recent study of children born before 24 weeks GA, 30% had a clinical diagnosis of ADHD at school age[16]. It also appears that the sociodemographic and genetic risk have less impact in infants born preterm. Instead, as for ASD, clinical risk factors are of importance[190].

1.6 MECHANISMS

At preterm birth, the maternal-fetal dyad is disrupted, and the immature infant faces extra-uterine challenges during a period of critical maturation, with undeveloped organs and without the mother's support to uphold metabolic equilibrium. Thus, several factors necessary for normal development are significantly altered in the preterm infant compared to the corresponding period during fetal life. The maturing preterm brain may be exposed to inflammation, hyper/hypoxia, and stress during a time of both nutritional and metabolic challenges. As a reference, the newborn brain following term birth requires well above 60% of the total energy intake[191].

1.6.1 CARBOHYDRATE METABOLISM

In the intrauterine environment, the primary factors influencing the regulation of glucose, insulin, and overall fetal growth is placental function and glucose control in the mother[192]. Under normal physiological conditions, the fetus does not produce glucose endogenously[193] but is dependent on delivery over the placenta. There is a linear relationship between maternal glucose levels and fetal glucose levels[193,194]. In contrast, insulin and glucagon do not cross the placental barrier[195]. Thus, the production of these substances relies on fetal activity. Studies have shown an essential role of insulin in fetal growth; this relationship is especially prominent at 29-40 weeks GA and insulin is also a determinator of birth weight[193,196].

1.6.1.1 PRETERM GLUCOSE REGULATION

The exact impact of preterm birth on glucose and insulin functions and interactions are largely unknown. The extremely preterm infant depends on continuous glucose infusions, but unlike in adults, endogenous glucose production is not suppressed by exogenic glucose supplementation. Following preterm birth, insulin levels are most commonly reported to be low, and pancreatic production is increased after the initiation of oral nutrients[197]. In extremely preterm infants, insulin resistance and a relative insulin deficiency are common features of the neonatal period. The first is related to the low volumes of skeletal muscles and adipose tissue, which are essential for sufficient peripheral glucose uptake[198]. Other contributing factors might be the administration of glucocorticoids and inotropic drugs, which suppress insulin secretion and affect insulin resistance[199]. Also, hepatic regulation of endogenic glucose homeostasis might fail following hepatic insulin resistance[200,201]. The compensating mechanism of increased insulin production may fail, thus

resulting in a relative insulin deficiency. This has been suggested to be a result of the immaturity of β-cells, but infants born extremely preterm with hyperglycemia also have faulty processing of pro-insulin to insulin[202].

1.6.1.2 HYPERGLYCEMIA

During the first 2 postnatal weeks, hyperglycemia is seen in around 88% of infants with a birth weight <1000 grams[203], and it is inversely related to GA and maturity[204]. However, in research and clinically, an absolute threshold for hyperglycemia is not agreed upon[205]. This is likely due to the lack of an exact threshold for negative long-term effects. The underlying causes of hyperglycemia are likely multifactorial. Increased glucose levels have been associated with excessive glucose infusions, steroid treatment, respiratory distress syndrome, sepsis, IVH, and reduced white matter[206-209]. More specifically, several independent associations between hyperglycemia, ROP, and IGF-1 have been described[210,211].

1.6.2 GROWTH FACTORS AND THE PRETERM BRAIN

1.6.2.1 INSULIN-LIKE GROWTH FACTOR 1

IGF-1 is a crucial regulator in pre- and postnatal neurodevelopment[212]. Preterm birth results in low serum concentrations of IGF-1 compared to intrauterine levels[212]. IGF-1 is a 70 amino acid protein with a molecule weight of 7.5kD. It is a mediator in the somatotropic axis and predominantly, the protein is synthesized in the liver, but also produced by all cell types in the brain. Around 99 % of the IGF-1 molecules are circulating in the body as a complex with one of at least 7 IGF binding proteins (IGFBPs) and a third protein labeled acid-labile subunit. Around 80% is bound to IGFBP-3, which extends the half-life of IGF-1 by decreasing proteolysis. The IGF-1 molecule binds to distinct receptors, mainly the IGF-1 R, a membrane-bound receptor consisting of 2 α-subunits and one β-subunit, structurally resembling a tyrosine-kinase family receptor. Downstream mechanisms activate the PI3K/AKT/MAPK pathways[213].

1.6.2.1.1 IGF-1 and the retina

IGF-1 is a known regulator of retinal development[214]. Previous studies indicate a role of IGF-1 in vascular growth in an experimental model of ROP as a

permissive factor in VEGF-activated endothelial cell proliferation which occurs during initial ROP phases[213]. In the preterm infant, decreased circulating IGF-1 levels have been linked to ROP development[213].

1.6.2.1.2 IGF-1 and the brain

In rats, IGF-1 reaches peak expression in the brain in the perinatal period with ongoing neurogenesis in several brain areas such as cerebellum, hippocampus, and olfactory bulb[215]. After the completion of this phase of neuronal proliferation, IGF-1 expression decreases[216]. The expression of the IGF-1 receptor is located in cortical grey matter, hippocampus, cerebellum, hypothalamus, and spinal cord, and the expression decreases following term birth. Overall, locally produced and secreted IGF-1 seems to play an important role in neurodevelopment.

IGF is a highly mitogenic protein improving cell survival, proliferation, migration, and growth. IGF-1 also impacts myelination, plasticity, and formations of synapses[212]. In the early development of the brain, IGF-1 promotes glucose uptake in neurons[217]. It is involved in all phases of neuronal maturation. In vitro studies have shown enhanced neuronal progenitor cell proliferation and maintenance and an increased number of neurons produced from neural stem cells after exposure to IGF-1[218]. It is also established that IGF-1 promotes subsequent steps of the differentiation of neurons, astrocytes, and OLs[219].

1.6.2.1.3 IGF-1 in the preterm infant

In preterm infants, decreased levels of IGF-1 are associated with poor growth[220]. In term infants, higher levels of IGF-1 in the umbilical cord are associated with increased fetal body size[221]. Further, associations between low levels of IGF-1 and IVH, ROP, bronchopulmonary dysplasia (BPD), and necrotizing enterocolitis (NEC) have been documented in preterm infants[222]. Low IGF-1 levels are also linked to altered brain volumes[223] as well as unfavorable neurodevelopmental outcomes at 2 years of age[224] in preterm infants. In a recent randomized trial investigating the role of exogenous IGF-1/IGFBP-3 treatment in extremely preterm infants, a tendency toward fewer IVH was observed[225].

1.6.2.1.4 IGF-1 and autism

A few studies have presented data indicating a link between autism and dysregulated IGF-1/PI3K/AKT/mTOR signaling[226]. IGF-1 levels are also

altered in an age-dependant manner in children with ASD[227-229]. In addition, IGF-1 has neuroprotective functions in the immature brain following neuroinflammartion[230,231] which in turn has been associated with autism-like behaviour.

1.6.2.2 BRAIN-DERIVED NEUROTROPHIC FACTOR

Levels of Brain-Derived Neurotrophic Factor (BDNF) are low following preterm birth[232]. BDNF is of central importance during different neurodevelopmental phases, with an important role in axon outgrowth, synaptic formation, stabilization, and transmission, as well as neural plasticity[233-236]. Further, BDNF has neuroprotective properties following oxidative injury as well as excitotoxic injury[237,238]. High expression of BDNF is found in the hippocampus, amygdala, cerebral cortex as well as cerebellum[239,240]. Further, there is a possible link between IGF-1 and BDNF function[241]. In preterm infants, low endogenous BDNF levels have been linked to unfavorable neurodevelopmental outcomes in early childhood[242].

1.7 MARKERS FOR NEUROVASCULAR INJURY

Early biomarkers for neurodevelopmental disorders in preterm infants would be of great importance for early diagnosis and thus enabling adequate support and therapeutic interventions. In the adult population, several brain injury markers are in clinical use for diagnosis, assessment of progression of disease, and evaluation of therapeutic interventions[243-245]. No such markers are in clinical use in preterm infants, and most studies in the neonatal setting have focused on term infants[246] or preterm infants with major brain injuries while few have focused on neurodevelopmental outcome. In term infants, elevated brain injury markers with association to asphyxia and severity of hypoxic-ischemic encephalopathy have been found in blood and cerebrospinal fluid (e.g., tubulin-associated unit [Tau], S100B, and neuron-specific enolase-[NSE])[247-253]. In this thesis, we focused on neurofilament light (NfL), a brain-specific peptide used as a marker for neuro-axonal injury, and glial fibrillary acidic protein (GFAP), a marker for astroglial cell injury.

1.7.1 NEUROFILAMENT LIGHT

NfL is a brain-specific peptide that is part of the neuronal cytoskeleton and used as a marker for axonal injury as it can be detected in peripheral blood as well as cerebrospinal fluid. In adults, it is used to evaluate the severity and clinical course of neurodegenerative diseases[243-245], and hypoxic and traumatic brain injuries[254,255]. In term infants, increased NfL has been associated with asphyxia, hypoxic-ischemic encephalopathy, and abnormal brain MRI[248,256]. Data on NfL in preterm infants are sparse, but an inverse relationship between circulating NfL and GA has been observed[257]. Recent studies also report an association between high levels of NfL and severe peri-/intraventricular hemorrhage/infarction in preterm infants as well as later poor motor outcome or death[257,258]. The same research group observed an increase in serum NfL levels during the first week of life[257].

1.7.2 GLIAL FIBRILLARY ACIDIC PROTEIN

GFAP is the main interfilament of the most abundant cell in the brain, the astrocyte. Following astrocyte death it is released into serum and has been used as a prognostic marker and to evaluate the course of neurodegenerative diseases in adults[259,260]. In comparison with NfL, GFAP seems to be circulating in peripheral blood for shorter periods in adults following brain injury and a detection span of hours rather than weeks has been suggested[261,262]. In term infants, GFAP has been suggested as a brain injury marker[263], but cord blood GFAP did not predict asphyxia, grade of encephalopathy or outcome in a recent study[264]. As for NfL, studies evaluating GFAP levels in preterm infants are few. A recent case-control study investigating the potential of GFAP as a biomarker for IVH or PVL in infants born preterm during the first 3 days of life did not show any elevated serum levels in the IVH/PVL group[265]. One study reported elevated levels of serum GFAP days 1-4 in low birth weight infants with periventricular WMI[266].

2 AIM

Preterm infants are at considerable risk of altered brain development, neurovascular injury and neurodevelopmental disorders. Endogenous levels of growth factors, involved in important neurodevelopmental processes, such as IGF-1 are low following preterm birth. Additional studies are required to elucidate the exact role of IGF-1 in the preterm infant. Further, the clinical benefits of identifying infants at high risk of altered neurodevelopment would be high, but the clinical usefulness of biomarkers used in the adult population, such as the axon-specific NfL, has not been studied in the preterm population.

The overall aim of this thesis was to investigate mechanisms and potential markers of abnormal neurovascular development and injury in preterm infants. The specific aims were to investigate associations of endogenous growth factors, such as IGF-1, and systemic brain injury biomarkers with abnormal brain development, neurovascular injury, and long-term adverse outcomes in preterm infants.

The specific aims of each paper were as follows

Paper I To investigate the relationship between hyperglycemia, the growth factor IGF-1, and the development of the neurovascular eye disease ROP in preterm infants and in an experimental model.

Paper II To evaluate longitudinal serum levels of brain injury biomarkers NfL and GFAP in preterm infants, and to explore links between these biomarkers and neonatal morbidities as well as neurodevelopmental outcomes at 2 years of age.

Paper III To investigate the connection between IGF-1 and brain development by utilizing brain volume segmentation at TEA MRI examinations in extremely preterm infants.

Paper IV To evaluate early postnatal levels of IGF-1 and NfL in association with long-term neurodevelopmental outcomes, including cognitive, motor, and neuropsychiatric diagnoses.

3 PATIENTS AND METHODS

Data from 3 clinical cohorts were utilized in this thesis. In addition, an experimental model for ROP was included in *Paper I*.

3.1 STUDY POPULATION

3.1.1 THE DONNA MEGA COHORT (*PAPERS I-IV*)

The Donna Mega cohort includes infants born extremely preterm at The Queen Silvia Children's Hospital, Gothenburg, Sweden 2013-2015. In total, 138 extremely preterm infants were born during the study period and 90 infants were included in the final study cohort with a mean (SD) GA at birth of 25.4 (1.4) weeks. Thirty-nine infants (43.3%) were females. The primary aim of the original randomized trial was to investigate the effect of a parenteral lipid emulsion containing fish oil on ROP, and the main findings with detailed description of the study inclusion process were published by Najm *et al.* 2017[267]. Infants were randomized to either Clinoleic®, or SMOFlipid®, MRI was performed at TEA, and children were examined at 2 years corrected age using Bayley scales of infant development (BSID)-III and at 5.5 years chronological age using Wechsler Preschool and Primary Scale of Intelligence (WPPSI)-IV and Movement Assessment Battery for Children (MABC)-2.

3.1.2 THE LUND COHORT (*PAPERS I-II*)

The Lund cohort comprises 74 infants out of 169 infants born before 32 weeks GA treated at the Skåne University Hospital, Lund, Sweden 2005-2007. The mean (SD) GA at birth was 27.1 (2). Thirty-five infants (47%) were females. Follow-up was performed at 2 years corrected age using BSID-II.

3.1.3 THE GOTHENBURG/UPPSALA COHORT (*PAPER II*)

The Gothenburg/Uppsala cohort includes 84 infants born <32 weeks GA 1999-2002 with a median (range) GA at birth of 27.2 (23.0-31.8) weeks. Forty-four (52%) infants were female. Seventy infants were admitted to The Queen Silvia Children's Hospital, Gothenburg, Sweden, and 14 infants were treated at Uppsala University Hospital, Uppsala, Sweden.

3.1.4 EXPERIMENTAL RETINOPATHY MODEL (*PAPER I*)

The oxygen-induced retinopathy mice model of ROP was initially described by Smith *et al.* in 1994 and developed further during later years[268,269]. It is designed to study retinal development and mimics the immature state of the retinal blood vessels following preterm birth[270]. The model allows for the study of ongoing postnatal development of the retinal vasculature and underlying molecular mechanisms, isolated from the influence of other developmental processes[271]. The model is schematically described in **Figure 7**. In short, the model encompasses both a quantifiable vaso-obliterative phase, corresponding to the first phase of ROP, and a second phase with neovascularization peaking at approximately postnatal day 17[269].

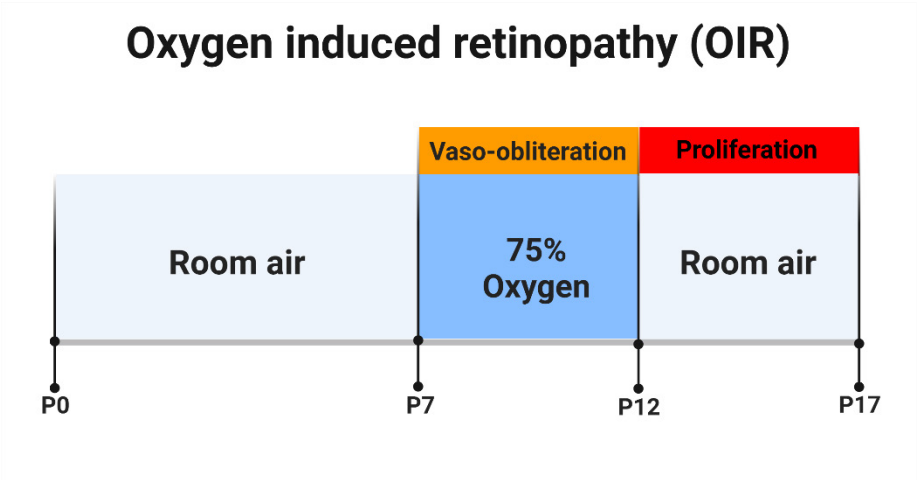

Figure 7. *Schematic overview of the oxygen-induced retinopathy (OIR) model, mimicking the course of ROP in the preterm infant. P: postnatal day. Created with BioRender.com.*

3.2 ETHICAL PERMITS

The Donna Mega cohort (Papers I-IV)
The blood sampling, MRI examinations and collection of clinical data were covered by ethical permit issued by the Regional Ethical Review Board in Gothenburg, Sweden (application Dnr 303-11, approved 2011-09-06).

The Lund cohort (Papers I-II)
The blood sampling, and collection of clinical data were covered by ethical permit issued by Regional Ethical Review Board in Lund, Sweden (application Dnr LU 87-03, approved 2003-02-12).

The Gothenburg/Uppsala cohort (Paper II)
This study was approved by the Ethical Review Board at Uppsala University, Sweden (Dnr 99033, approved 1999-03-08) and the Regional Ethical Review Board of Gothenburg, Sweden (application Dnr 547-98, approved 1998-11-16).

Experimental ROP model (Paper I)
The experimental ROP model (oxygen-induced retinopathy in mouse) was performed at Boston Children's Hospital/Harvard Medical School, Boston, MA, USA. The study was executed in agreement with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The study was authorized by the Boston Children's Hospital Institutional Animal Care and Use Committee (19-04-3913R).

3.3 MEASUREMENTS AND EVALUATIONS OF OUTCOME

3.3.1 RETINOPATHY OF PREMATURITY

Screening for ROP was performed according to Swedish national guidelines including infants born at <32 weeks GA prior to July 2012 and <31 weeks GA from July 2012 and onwards. Ophthalmological examinations started at postnatal age 5-6 weeks and at the earliest at postmenstrual age 31 weeks. Screening was performed by dilating the pupils during the screening period until approximately TEA, when the retinal vasculature had grown out in the periphery (biweekly to twice a week, based on the presence and severity of ROP). Generally, infants undergo a median of 7 examinations, a number that increases with decreasing GA.

3.3.1.1 EXPERIMENTAL OXYGEN-INDUCED RETINOPATHY MODEL

In the oxygen-induced retinopathy mice model of ROP, a hyperglycemic, hypoinsulinemic state was induced by streptozotocin destruction of β cells in the pancreas. The C57BL/6 mouse was used. Following the induction of hyperglycemia, mice pups were exposed to recombinant IGF-1 and at the end of the study hepatic samples were taken and prepared for Ribonucleic Acid (RNA) analysis according to a strict protocol.

3.3.2 NEURODEVELOPMENTAL EVALUATION

Since 2015 all Swedish infants born <28 weeks GA are included in a national follow-up program with the aim of early identification of neurodevelopmental deviations and early referral for further evaluation[272]. Mandatory visits with reports to the neonatal national patient registry (Swedish Neonatal Quality Register [SNQ]) are scheduled at 2 years corrected and 5.5 years chronological age and all infants undergo brain stem audiometry and eye examination upon discharge from neonatal care. The following standardized tests and validated questionnaires are included in the program.

3.3.2.1 AT 2 YEARS CORRECTED AGE

3.3.2.1.1 Bayley scales of infant development-III

Developmental evaluation including cognitive, language, and motor scales performed by a trained psychologist. In study cohorts prior to the implementation of the national program BSID-II was used. Scores are not immediately comparable between BSID-II and BSID-III, and adjustments of cut-off scores are needed[273,274].

3.3.2.1.2 Modified Checklist for Autism in Toddlers (M-CHAT)

M-CHAT is a two-stage parent questionnaire used to screen for early signs of autism (www.m-chat.org).

3.3.2.2 AT 5.5 YEARS CHRONOLOGICAL AGE

3.3.2.2.1 Wechsler Preschool and Primary Scale of Intelligence IV (WPPSI-IV)

An intelligence test for children up to 7.5 years with subscales used to calculate verbal, performance and full-scale IQ. Performed by a trained psychologist.

3.3.2.2.2 Movement Assessment Battery for Children 2 (MABC-2)

Standardized test of multiple motor domains performed by a trained physiotherapist. The test can be used to diagnose DCD with a cut-off at $\leq 5^{th}$ percentile for definite and $\leq 15^{th}$ percentile for suspect motor impairment.

3.3.2.2.3 The Strengths and Difficulties Questionnaire (SDQ)

Brief emotional and behavioural questionnaire for parents and teachers.

3.3.3 VOLUMETRIC SEGMENTATION AT MAGNETIC RESONANCE IMAGING

MRI-based volumetric segmentation of the brain of the extremely preterm infant is a complex process with specific challenges compared to the corresponding examination of the mature brain. Challenges include low contrast-to-noise ratio and signal-to-noise ratio, in addition to high variability in size and morphology during this intensive phase of development. In *Paper*

III, volumetric segmentation was performed on T2-weighted images, which were merged into a 3D image volume for each infant. The regional anatomical volumes were retrieved based on a volumetric segmentation procedure, previously specified by Makropoulos *et al.* in the Developing human connectome project[275]. The regional volumes were classified based on to the atlas presented by Gousias *et al.*[276]. The automatic anatomical segmentation was atlas-based, performed by utilizing the Developing brain Region Annotation with Expectation-Maximization (DrawEM), which is a module of the Medical Image Registration Toolkit[136,275]. Following a quality scoring protocol, insufficiently defined volumetric segmentations were removed. The final step included a merging of anatomical subregions into cortical grey and deep grey matter, white matter, and cerebellum, as well as total brain volume (after removing cerebrospinal fluid volume and ventricular volume), **Figure 8**.

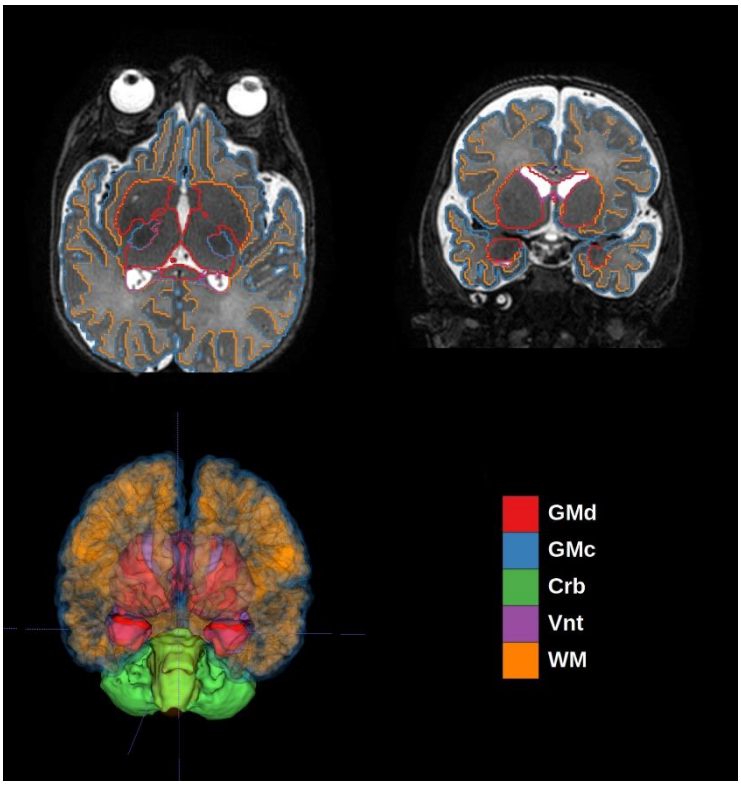

Figure 8. *Volumetric segmentation of the neonatal brain. GMd: grey matter deep, GMc: grey matter cortical, Crb: cerebellum, Vnt: ventricles, WM: white matter*

3.3.4 BLOOD SAMPLING REGIMES

Study cohorts in this thesis include very preterm and extremely preterm infants and serial blood samples were retrieved according to cohort-specific regimes. An overview of blood sampling regimes is shown in **Figure 9**.

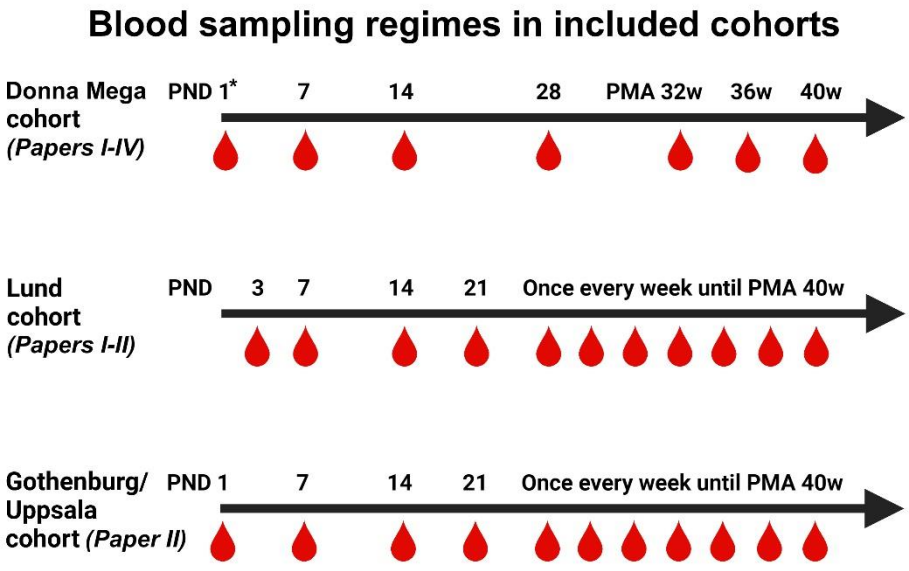

Figure 9. *Blood sampling regimes in study cohorts. *Cord blood samples also available. PND: postnatal day, PMA: postmenstrual age, w: weeks. Created with BioRender.com.*

3.3.5 LABORATORY ANALYSES

Details on laboratory analyses used are presented in each paper.

3.3.5.1 MEDIAGNOST, RADIOIMMUNOASSAY

Radioimmunoassays (RIAs) are traditional immunological assays where radio-labelled recombinants of the analyte (tracers) are used for detection. In Mediagnost IGF-1 RIA, a secondary antibody is used to precipitate complexes of IGF-1 and primary antibodies, which allows unbound tracers to be removed. Specific for Mediagnost IGF-1 RIA is an initial dissociation of IGF-1 from the carrier protein IGFBP-3 by reducing the pH in the sample. An excessive amount of IGF-2 with higher affinity for IGFBP-3 is added to keep IGF-1 free for analysis.

3.3.5.2 SIMOA

Simoa® (Quanterix) is a new generation, semi-automatic and ultrasensitive immunological assay. A primary antibody is utilized to trap the analyte of interest to paramagnetic beads and a secondary antibody attached to a fluorophore is used for detection. An electromagnetic field trap complexes of bead, antibodies and antigens in microwells (one bead per well) on a disc and fluorescent substrate is used for amplification and detection of complexes.

3.3.5.3 HANDLING OF ANALYTICAL ERRORS

All analytical methods include errors related to the assay and/or the performance. Automation of analytical steps partially handled variation induced by laborants. In addition, aliquots of one or more pooled samples were used to calculate the inter-assay variation between different plates while intra-assay variation was estimated by the coefficient of variation of multiple (e.g. duplicate) samples on the same plate.

3.3.6 COMMENTS ON SELECTED STATISTICS

Details on statistical methods are presented in each paper.

3.3.6.1 AREA UNDER THE CURVE *(PAPER II, III, IV)*

As a measure of longitudinal endogenous exposure, area under the curve (AUC) was used in *Papers II-IV*. AUC was retrieved by utilizing the trapezoidal rule[277]. AUC provides a comprehensive analysis of exposure under highly variable clinical conditions with irregular sampling but is less usable for identification of single sample biomarkers with defined cut-off values.

3.3.6.2 REGRESSION MODELS

Both binary and linear regression models were utilized depending on outcome variables. Adjustment for confounding factors is of specific importance in extremely preterm infants as clinical variables including GA and sex have profound effects on any morbidity or outcome within the group. In *Paper I,* mixed model regression analysis was performed. In *Paper II* and *Paper IV,* binary regression models were utilized. In *Paper III*, linear regression models were utilized.

4 RESULTS AND DISCUSSION

4.1 INSULIN-LIKE GROWTH FACTOR 1 AND ITS RELATION TO HYPERGLYCEMIA AND RETINOPATHY OF PREMATURITY (*PAPER I*)

In *Paper I,* we showed that high glucose levels were linked to low IGF-1 levels in extremely preterm infants and that high glucose levels were associated with ROP development. In the hyperglycemic/hypoinsulinemic oxygen-induced retinopathy mice model of ROP, these findings were confirmed as diminished insulin signaling was associated with decreased hepatic IGF-1 output, as well as retinal neovascularization. In addition, exogenous IGF-1 had beneficial effects on retinal vascularization, suggesting a mechanistic role for IGF-1.

In total, 117 infants were included with a mean (range) GA of 25.4 (22.7–27.9) weeks at birth. We showed that plasma glucose levels were inversely related to GA at birth, r = –0.648, P <0.0001. Infants with the highest plasma glucose levels, presented as tertiles, had mean glucose values that correlated with amount of parenteral glucose (r = 0.67, P <0.0001). Infants with the highest glucose had lower longitudinal IGF-1 serum levels when compared to infants in the low and intermediate glucose tertiles at postnatal day 28 (P = 0.038 and P = 0.03). Infants in the high plasma glucose tertile also developed ROP to a larger extent than infants in the low glucose tertile (87% vs. 49%), and the prevalence of severe ROP was higher in the high tertile (71% versus 32%).

In the experimental oxygen-induced retinopathy mice model of ROP, hyperglycemia induced lower hepatic expression of IGF-1 (P <0.0001) at P12 and at P17 (P <0.0001). When exposed to recombinant human IGF-1, the physiological vascular regrowth increased and pathologic neovascularization decreased (P = 0.027 and P <0.0001 respectively).

An increasing number of studies indicate a specific role of IGF-1 in neurovascular injury and neurodevelopmental outcome[212]. The results in our study are in line with previously published clinical and experimental data[214,278-280]. The potential beneficial effect of IGF-1 on neurovascular morbidity was recently evaluated in a multicentre trial of exogenous rhIGF-1/rhIGFBP-3 administration in extremely preterm infants. Contrary to previous findings, the study showed no reduction in ROP development[281]. One possible explanation

provided by the authors included an increase in target oxygen saturation prior to study start, which could have promoted ROP and surpassing the role of IGF-1. In addition to decreased IGF-1 levels, high glucose could in itself affect ROP development[211]. In addition, excess glucose can, via the generation of more reactive oxygen species and/or by a defective scavenging system, lead to apoptosis and cellular dysfunction in the critically ill[282]. To establish a mechanistic role of IGF-1 in ROP development further studies are needed.

4.2 NEUROFILAMENT LIGHT IN VERY PRETERM INFANTS AND ITS RELATION TO RETINOPATHY OF PREMATURITY AND NEURODEVELOPMENTAL OUTCOME AT 2 YEARS CORRECTED AGE (*PAPER II*)

In *Paper II,* we found an immediate increase in serum NfL levels following preterm birth, with high levels persisting the first month of life before levelling out. Further, we showed that high NfL levels in the postnatal period associated with ROP and exploratively linked to unfavorable neurodevelopmental outcome at 2 years corrected age.

In total, 221 infants were included with a mean (SD) GA of 26.5 (2.1) weeks at birth. NfL serum levels increased following preterm birth and remained elevated for the first month, followed by a decline after 3 months of life, **Figure 10**. In the final binary regression model (including GA at birth, Apgar score at 5 minutes, birth weight standard deviation score, as well as the mode of delivery), NfL (AUC week 2-4) was linked to the development of any ROP (P <0.001) with an OR (95% confidence interval [CI]) of 4.8 (2.2-10.6). Further, in an explorative binary regression analysis, NfL (AUC week 2-4) was linked to lower BSID scores at 2 years corrected age (P = 0.01), with an OR (95% CI) per 10-unit NfL AUC increase of 1.07 (1.02-1.13). No associations were found between GFAP and any of the outcomes.

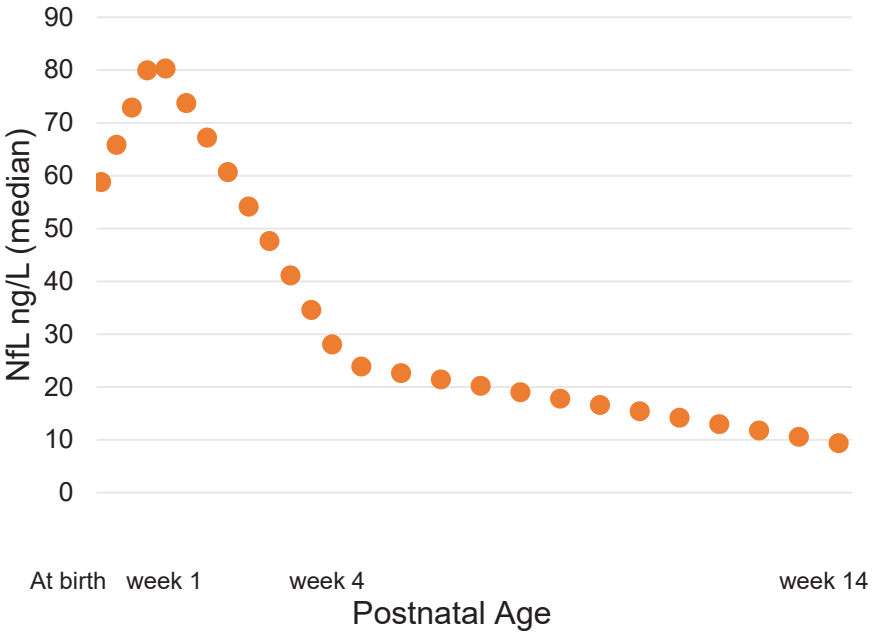

***Figure 10**. Simplified presentation of longitudinal serum levels of neurofilament light following very preterm birth.*

Published data on NfL in preterm infants are scarce, but our findings correspond to results from a previous study including more mature infants, as well as a recent publication investigating infants with moderate to severe IVH/PIVH which indicates a maturity-dependent decrease of NfL levels, as well as a link between early elevated serum levels of NfL and brain injury, poor motor outome at 2 years of age and mortality[257,283]. Although promising results have been shown in preterm infants and a clinical role of NfL is established in the adult, comprehensive prospective studies are needed to evaluate whether NfL might be a clinically relevant marker for brain injury and adverse outcome in the preterm setting.

4.3 NEONATAL INSULIN-LIKE GROWTH FACTOR 1 LEVELS AND BRAIN VOLUMES AT TERM EQUIVALENT AGE (*PAPER III*)

In *Paper III*, we showed that low postnatal levels of IGF-1 were associated with decreased total and regional brain volumes at term.

In total, 49 extremely preterm infants with a median (range) GA of 25.4 weeks (22.9–27.9) were included. IGF-1 levels in the first month of life (AUC week 1-4) were associated with increased total and regional brain volumes at term in univariate correlation, as well as in a regression model following adjustment for GA at birth and postmenstrual age at time of MRI scan (total brain volume [P <0.001, $\beta = 0.90$, $R^2 = 0.64$], white matter volume [$P = 0.007$, $\beta = 0.33$, $R^2 = 0.30$], cortical grey matter volume [$P = 0.002$, $\beta = 0.43$, $R^2 = 0.72$], deep grey matter [$P = 0.008$, $\beta = 0.05$, $R^2 = 0.41$], and cerebellar volume [$P = 0.006$, $\beta = 0.08$, $R^2 = 0.63$]). P-values, 95% CI of β illustrated in **Figure 11**. The results remained statistically significant after adjustment for significant brain injury, which was defined as IVH grade 3, PVHI, and/or abnormality classified by Kidokoro *et al.*[284]. No associations with brain volumes were found for BDNF, platelet-derived growth factor (PDGF), or VEGF.

In an explorative sub-analysis of the impact of GA, associations between endogenous IGF-1 levels and brain volumes were most prominent in the more mature infants (GA above 25 weeks or above median of the study group).

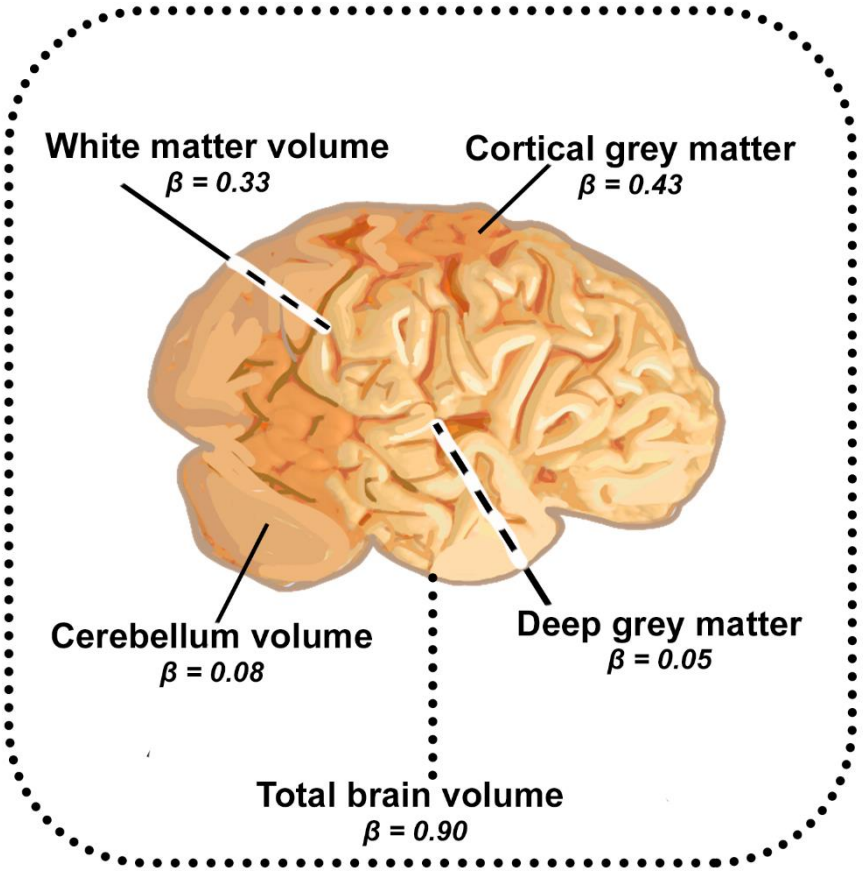

Figure 11. *Insulin-like growth factor 1 AUC postnatal week 1-4 and median/mean brain volumes at TEA in a regression model, adjusted for gestational age at birth and postmenstrual age at magnetic resonance imaging examination, 95% CI of β for total brain volume 0.41–1.39, white matter 0.09–0.56, grey matter: 0.17–0.70, deep grey matter 0.01–0.08 and cerebellar volume 0.02–0.13. All associations remained statistically significant following multiple adjustments. AUC: Area under the curve, CI: confidence interval, TEA: term equivalent*

These results are in line with the findings of Hansen-Pupp *et. al.* who demonstrated a similar association in less immature infants and linked early circulating IGF-1 levels to neurodevelopmental outcome at 2 years of age[223,224].

As discussed in this thesis, several potential factors could influence brain growth, and although cumulative evidence suggests a link between reduced brain volume at term and adverse neurodevelopmental outcomes, results of previous studies are not consistent[70,104,285]. A possible explanation is lack of standardized volumetric methods which may influence our comparisons with previous studies. We also found that the association between early postnatal levels of IGF-1 and brain volumes was more pronounced in more mature infants. Available data on neurovascular morbidity suggest that the function of IGF-1 might be threshold-related[213]. A certain level of circulating IGF-1, which is also related to GA and postnatal age, might be required for a more pronounced role of IGF-1 on brain development.

4.4 NEONATAL INSULIN-LIKE GROWTH FACTOR 1 AND NEUROFILAMENT LIGHT LEVELS IN RELATION TO NEURODEVELOPMENTAL OUTCOME AT EARLY SCHOOL AGE (*PAPER IV*)

In *Paper IV*, we investigated associations between early postnatal serum levels of IGF-1 and NfL and long-term neurodevelopmental outcome at early school age in extremely preterm infants. The importance and relevance of the findings are discussed in *Paper IV*.

4.5 SUMMARY

In summary, this thesis presents results that suggest that increased levels of IGF-1 are associated with improved brain development and may have a beneficial role in ROP development in extremely preterm infants, where endogenous levels are generally low. In addition, studies included in the thesis indicate that an early elevation of brain injury marker NfL is associated to ROP as well as poor long-term neurodevelopmental outcome. This is of particular interest as an increasingly high prevalence of neurodevelopmental disorders is reported as more immature infants survive and early reliable markers for adverse outcome are lacking.

5 GENERAL CONSIDERATIONS

Studying neurovascular development in extremely preterm infants present multiple challenges, including difficulties in obtaining representative samples at relevant time-points to reflect an ongoing process.

5.1 AVAILABILITY AND VARIABILITY OF STUDY SUBJECTS

There is an extreme heterogeneity within this patient group with individual pre-and postnatal exposures, insults, nutritional practices and therapeutic interventions. In addition, GA at birth, as well as postnatal age and postmenstrual age are likely to affect developmental processes as well as results of sampling and examinations with different implications at different developmental stages. One way to overcome these difficulties would be larger, prospective studies providing more data, but every large study in this rare group of patients requires careful consideration as it will obstruct other studies. This emphasizes the importance, from a scientific as well as an ethical perspective, of utilizing data already collected to answer new research questions. In our studies, this is reflected by the retrospective use of data and samples that were prospectively collected in the clinical studies included.

5.2 SELECTION AND TIMING OF SAMPLING AND IMAGING

Another important factor when conducting research in this clinical setting is to select easily accessible, well-defined, and measurable variables and short-term outcomes, preferably at standardized time points. A strength of this thesis is the availability of longitudinal blood sampling in several preterm cohorts. Still, different cohorts had different sampling regimes, sampling was dependent on clinical decisions to draw blood leading to deviations from the planned regimes and missing values from different time points. The use of AUC was an attempt to overcome these limitations but prevented us from defining single biomarkers at select time points for potential clinical use.

Short-term outcomes consisted of standardized ROP screening according to an established protocol in clinical use. MRI was performed as part of clinical

routine in some infants with added research images and as pure research examinations in others. Our studies are limited by families declining MRI due to long journeys or other reasons, and by poor image quality in a number of patients.

5.3 LONG-TERM FOLLOW-UP

A major strength of this thesis is the availability of long-term follow-up data collected prospectively with the use of standardized methods and with diagnoses of neurodevelopmental disorders and/or test results from almost all children up to school age. The importance of long-term follow-up is illustrated by our findings of NfL association with cognitive scores at 2 years of age and with other neurodevelopmental disorders at 5.5 years suggesting that causes of developmental delays and deviations become clearer over time.

5.4 THE MECHANISTIC IMPORTANCE OF INSULIN-LIKE GROWTH FACTOR 1

Despite the strong association of low circulating IGF-1 with neurovascular disease and neurodevelopment and in spite of the experimental study supporting a mechanistic role, IGF-1 may serve as a modulator and/or a marker for other biological processes of higher mechanistic importance. It should also be noticed that low levels of IGF-1 are associated with other severe neonatal morbidities such as BPD and NEC, that in themselves may influence brain development and outcome by other mechanisms.

To evaluate causal relationships in the preterm infant is not an easy task. Extremely preterm infants are exposed to intensive care, together with high risk of dysfunction of multiple organ systems. Following the introduction of new advanced analytic techniques, such as proteomics, and new assays, many studies of biomarkers and associative relationships to outcome are published, which in many cases contribute to completing the current knowledgebase. However, due to the complexity of especially brain development, the need for well-defined long-term follow-up routines, is crucial. The role of (inter)nationally accepted follow-up regimes, availability of databases for clinical and laboratory data, and quality registers should be emphasized in order to facilitate research and further improve neonatal care.

5.5 BIOMARKER CHALLENGES IN PRETERM INFANTS

In this thesis, we found an association between high postnatal levels of NfL and ROP in the neonatal period, as well as adverse neurodevelopmental outcomes later in childhood. The results are in line with the few studies that have previously addressed NfL in the neonatal setting. No markers for preterm brain injury or brain development are in clinical use. This may be explained by the complex and prolonged nature of the injury process. Longitudinal measurements, rather than a single value, may better reflect the complex pathophysiology of disturbed brain maturation but is of little help in identifying simple markers for clinical use. Larger, prospective studies are needed to validate our findings and explore the clinical relevance. Other factors complicating the search for relevant biomarkers are varying definitions of brain development as well as brain injury, small study groups with differing inclusion criteria, limited follow-up time as well as difficulties in retrieving long-term data. Our study is limited by brain volume measurements as the only short-term outcome regarding brain injury and brain development but strengthened by availability of long-term data.

6 CONCLUSIONS AND FUTURE DIRECTIONS

- High neonatal glucose levels were associated with low serum IGF-1 levels and ROP in preterm infants. IGF-1 may help prevent the neurovascular disease ROP, but results from intervention studies are hitherto not entirely conclusive.

- Low serum levels of IGF-1 during the first month of life in extremely preterm infants were associated with reduced brain volumes at TEA suggesting a protective role of IGF-1 in brain development.

- Serum levels of brain injury biomarker NfL were increased following very preterm birth, and elevated levels were associated with the development of ROP and adverse neurodevelopmental outcome in early childhood at 2 years corrected age. However, timing of sampling and cut-off values have not been established.

- Extremely preterm infants with high neonatal serum levels of brain injury marker NfL and low levels of IGF might be at increased risk of adverse neurodevelopmental outcomes at early school age.

These findings warrant further investigation and their clinical utility should be elucidated in larger prospective cohorts.

7 ETHICS

A wide range of ethical questions needs to be addressed and thoroughly considered when conducting trials involving critically ill, extremely preterm infants in intensive care. These infants represent the most fragile group of individuals within the neonatal population and continued research is crucial to reduce mortality and morbidity in this growing population.

7.1 THE DECISION TO ENROLL

An infant cannot decline participation or consider the benefits and disadvantages of a scientific study. Parental consent for trials among neonates presents particular problems, even when pregnancy, labour, and delivery are uncomplicated[286,287]. The decision regarding an infant's participation in a study lies solely with the parents. Parental consent is often required in close connection to preterm birth, an event that is related to considerable emotional stress[288]. Under the Helsinki declaration, constituted by the World Medical Association, a fundamental principle in human research is the right of self-determination and right to make informed decisions prior to participation. For these infants and children, this decision is made by the parents who need to be well informed to a make a decision on behalf of their child. In the studies of this thesis, both oral and written information was given by research staff familiar with all procedures, and written consent was required for participation. In addition, information was repeated throughout the studies, and research staff was available in the neonatal intensive care unit for any questions.

Further, an individual subject's welfare must always have priority over science and society's interests. In the United Nations Convention on the Rights of the Child article three, the child's best interests shall be a primary consideration[289]. In the studies included in this thesis, blood sampling regimes, examinations, and interventions were carefully evaluated to minimize discomfort, pain, and potentially harmful events. MRI examinations at TEA were performed without contrast medium and under light sedation as in clinical routine. Blood sampling was restricted to sampling from venous or arterial lines or in association with venous punctures for clinical purposes. ROP screening was performed as part of the clinical routine program. No procedures were considered as a medical risk although minor discomfort could not be excluded.

7.2 BLOOD SAMPLING IN THE NEONATAL SETTING

In recent years, the blood sampling regimes in neonatal intensive care units have been addressed. Recent studies suggest a 58% blood volume loss in extremely preterm infants due to clinical blood sampling the first 2 weeks of life followed by replacement transfusions[290]. Excessive blood sampling may lead to loss of important blood factors including fetal haemoglobin with a link to neonatal morbidity[291,292]. Currently, a multicentre randomized controlled trial with the aim of minimizing blood loss during the first weeks of life is ongoing in Sweden (ClinicalTrials.gov Identifier: NCT04239690). In the studies included in this thesis, blood sampling was restricted due to the low blood volume of the preterm infant, samples were used for multiple research questions in several studies and new methods requiring minute volumes were used.

7.3 EXPERIMENTAL ANIMAL RESEARCH

The potential benefit for science versus the animals' welfare must be thoroughly considered when conducting animal studies. It is essential to recognize the animal as a sentient being and do everything possible to maximize the animal's well-being and minimize harm. Novel strategies are being developed, and the role of animal research might be diminished in the future by, for example, using computational simulations and cell cultures. In light of the high risk of mortality, morbidity, and long-term sequels and the fact that biomarkers and mechanisms of abnormal development are largely unexplored in this patient group of prematurely born children, the potential clinical gain could justify animal studies according to guidelines for animal welfare in research (SJVFS2019:9, Saknr L 150). The animal research conducted in this thesis utilized an oxygen-induced retinopathy mice model of ROP with well established methods, and executed in agreement with the Association for Research in Vision and Ophthalmology Statement (ARVO) and US legislations, and were approved by the Boston Children's Hospital Institutional Animal Care and Use Committee. As stated by the Norwegian Committee for Research Ethics in Science and Technology on the matter, "Our treatment of animals, including the use of animals in research, is an expression of our attitudes and influences us as moral actors"[293].

8 ACKNOWLEDGMENTS

I hereby would like to highlight the importance of collaborative efforts when performing research. I would not have come anywhere without the wonderful help of many extraordinary others who have shared their invaluable knowledge. I cannot enough express my gratitude to all of you for taking the time and effort to help me along with this, but I will try to put it in writing below.

First, thank you to **all the patients and parents** at Queen Silvia's Children's Hospital, Skåne University Hospital, and Uppsala University Hospital, who accepted to take part in these studies and willingly participated in the follow-up.

I thank every single person involved in these studies, **physicians**, **nurses**, **assistant nurses**, **janitors**, **administrative staff**, and all others involved in one way or another.

My main supervisor, neonatologist Dr **Karin Sävman**, for all the incredible support, encouragement and both scientific and clinical knowledge that you have shared. Thank you for your wise thoughts and invaluable aid in this thesis, and also for being the first to really introduce me to the field of neonatology and brain development. I doubt there could be a better teacher, and I hope our journey will continue.

My co-supervisor, associate professor **Chatarina Löfqvist** for your thorough research knowledge spanning over many fields, your openness, and ability to orientate among difficult obstacles. You have been instrumental in many different stages of this work, but also on the first steps on my research journey in general, supervising me during my master thesis at medical school, and my first publication several years ago.

My co-supervisor, neonatologist Dr **Matteo Bruschettini** for your continuous support, wise advise, hands-on research help, and for important angles of discussion of science, issues of highest importance during thesis writing.

Professor **David Ley** for being a brilliant researcher and for continuously being a role model, both in research and in life. Thank you for all your help, for creating the best of scientific environments, and for inviting me to exciting projects together with the members at your fantastic lab.

PhD student, MSc **Ulrika Sjöbom**, for being a great co-writer, colleague, and for her fantastic laboratory knowledge.

Professor **Lois Smith** for her unparalleled knowledge, illuminating discussions and for taking care of me at her lab and in Boston.

Dr **Bertan Cakir** for his expertise in the oxygen-induced-retinopathy model, and his great thoughts and ideas as the first author in *Paper I.*

Dr **Ingrid Hansen-Pupp** for her clinical and scientific expertise in the field of neonatology.

Professor **Isabella M. Björkman-Burtscher** for her guidence and great knowledge in the field of MRI.

Professor **Rolf A. Heckemann** for performing the volumetric segmentations, for his thorough knowledge and his strive for further improvement of advanced methods.

Statisticians **Aldina Pivodic** and Professor **Staffan Nilsson** for contributing with statistical expertise and always helping out when needed in a very uncomplicated way.

Dr **Anna-Lena Hård**, for her very wise thoughts and valuable suggestions.

Associate professor **Anders K. Nilsson**, for his tremendous knowledge in science and continuous drive for further development.

Nurse **Carola Pfeiffer Mosesson** for continuously being supportive to everyone involved, and for helping out whenever help is needed.

In addition, my lovely, but also slightly crazy friends (historically, now of course well-functioning), lawyer **David Schreiber**, designer **Gustaf Gunér**, doctor **Fredrik Holm,** and psychologist **Robert Jallinder**.

My sisters **Josefin Nerme** and **Wilma Hellström** for being wonderful siblings.

My soon to be wife **Matilda**, for continuously encouraging me whole-heartedly (also during the Christmas holidays). Thank you for supporting my endeavours in life.

Finally, mom professor **Ann Hellström** and dad professor **Mikael Hellström**. I really cannot thank you enough for all your wise thoughts, guidance,

encouragement, and vast knowledge, both regarding complex challenges in the field of science, and on simpler (but perhaps still as important) everyday subjects in the humble existence of a human being. You are the best parents for whom one could ever hope for. Thank you for teaching me what really matters in life.

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