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Sveinsdottir, Kristbjorg

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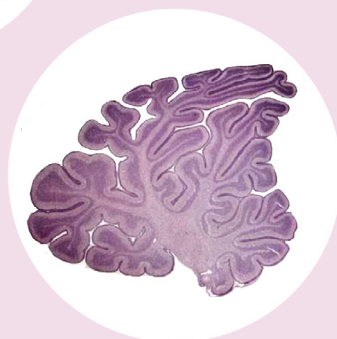
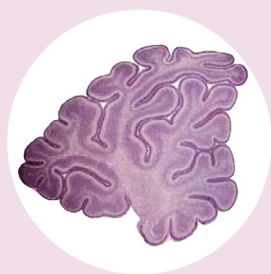
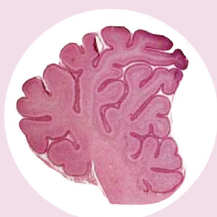
PO Box 117
221 00 Lund
+46 46-222 00 00

Cerebellar development and very preterm birth

interactions with neonatal events

KRISTBJÖRG SVEINSDÓTTIR

DEPARTMENT OF CLINICAL SCIENCES | FACULTY OF MEDICINE | LUND UNIVERSITY



Cerebellar development and very preterm birth – interactions with neonatal events

Kristbjörg Sveinsdóttir



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DOCTORAL DISSERTATION

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<p>Abstract</p> <p>The cerebellum is an important part of the brain and in very preterm infants the cerebellum is smaller at term equivalent age compared to that of infants born at term.</p> <p>Hypothesis and aims: The general hypothesis is that preterm birth <i>per se</i> causes cerebellar underdevelopment and that certain events following preterm birth, either complications or treatments, can aggravate the underdevelopment. The general aim is to evaluate how preterm birth in association with potentially damaging events <i>i.e.</i>, intraventricular hemorrhage (IVH), the neurovascular disease retinopathy of prematurity (ROP) and the widely used adenosine antagonist caffeine, can affect the cerebellar development.</p> <p>Methods: <i>Paper I.</i> Postnatal growth, cerebellar external granular layer (EGL) development and circulating IGF-1 levels were evaluated following preterm birth in rabbit pups and compared to that of term pups to evaluate if the preterm rabbit pup model is suitable for future cerebellar studies. <i>Paper II.</i> The distribution and effect of cell-free hemoglobin (Hb) on cerebellar development was evaluated following IVH in preterm rabbit pups and compared to preterm pups without an IVH. Additionally the effect of the cell-free Hb scavenger Haptoglobin (Hp) was evaluated. <i>Paper III.</i> The relationship between any stage of ROP, brain volumes and developmental outcome was evaluated in very preterm infants. <i>Paper IV.</i> The effect of caffeine on cerebellar development and the Insulin-like growth factor 1 (IGF-1) system was evaluated in preterm rabbit pups given enterally administered caffeine and compared to control preterm pups.</p> <p>Results: <i>Paper I.</i> Mean weight and circulating levels of IGF-1 were significantly lower in preterm pups at all time-points (all $p < 0.05$). Postnatal weight correlated with circulating IGF-1 ($r^2 = 0.89$) independently of gestational age at birth and postnatal age. Proliferation was reduced in preterm pups at postnatal day 2 (P2) compared to term pups ($p = 0.01$). Purkinje cells in the preterm pups exhibited reduced calbindin staining at P0 ($p = 0.003$), P2 ($p = 0.004$) and P5 ($p = 0.04$) compared term group. <i>Paper II.</i> IVH was associated with a decreased proliferative (Ki67 positive) portion of the EGL, delayed Purkinje cell maturation, and activated microglia in cerebellar white matter. Immunolabeling at P0 in pups with IVH, demonstrated a widespread presence of cell-free Hb in all cerebellar layers. Intraventricular injection of Hp resulted in a distribution of Hp corresponding to that of cell-free Hb in the cerebellar layers and a partial reversion of the damaging effects observed following IVH. <i>Paper III.</i> Infants with any stage of ROP had lower unmyelinated white matter volume (UWMV) ($p < 0.001$), cerebellar volume ($p < 0.001$), mental developmental index ($p < 0.001$) and psychomotor developmental index ($p = 0.002$) compared to infants without ROP. <i>Paper IV.</i> Caffeine treatment did not affect weight development, serum IGF-1 or hepatic expression of mRNA for IGF-1 at any given time-point. Cerebellar densities of IGF-1R, calbindin positive Purkinje cells and Ki67 positive proliferating cells were not affected by caffeine exposure.</p> <p>Conclusions: The preterm rabbit pup model exhibits characteristics relevant to human preterm birth and is appropriate for further studies on preterm birth and cerebellar development. IVH in the preterm rabbit pups is followed by extensive deposition of cell-free Hb in cerebellar layers and associated with microglial activation, reduced neuronal proliferation and Purkinje cell maturation. Intraventricular injection of the scavenger Hp partially blocked these effects suggesting that cell-free Hb is causal in cerebellar impairment following IVH. Development of any stage of ROP was associated with reduced cerebellar volume and UWMV and impaired neurodevelopmental outcome at 2 years corrected age. Enteral administration of caffeine to preterm rabbit pups did not reveal effect on growth or the closely related IGF-1 system in preterm rabbit pups and did not affect key neuronal maturation in the cerebellar EGL. It did increase survival.</p>		
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Kristbjörg Sveinsdóttir



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Small

Small soul
Whose cry broke through
Immature lungs
Immature throat
You were born to fight

Small arms
Who flail against the world
Beating fists
Beating heart
You were born to fight

Small body
That yearns to grow
Beeping lights
Beeping alarm
You were born to fight

Small mouth
That aches to feed
Searching lips
Searching eyes
You were born to fight

Small soul
Whose cry broke through
You can breath
You can sing
Home

Table of Contents

List of papers	9
Other publications not included in the thesis.....	10
Abbreviations.....	11
Summary.....	12
The thesis at a glance.....	14
Preface	15
Background	17
Very preterm birth	17
Definitions	17
Survival.....	17
Major complications.....	17
Neurodevelopment in preterm infants	18
Cerebellum.....	19
The role of the cerebellum.....	19
Cerebellar development.....	19
Key factors in cerebellar development	21
The effect of preterm birth on cerebellar development	23
Cerebral intraventricular hemorrhage - IVH	26
IVH and the cerebellum.....	27
The damaging effect of cell-free hemoglobin in the brain	27
Hemoglobin clearance – the haptoglobin system	28
Retinopathy of prematurity.....	29
Risk factors.....	30
ROP and long term complications.....	31
Caffeine	33
The Insulin-like growth factor system.....	35
Developmental similarities in the retina and cerebellum	37
The preterm rabbit pup model	38
Hypothesis and aims	41
Material and methods.....	43
Preterm animals	43

The preterm rabbit pup model	43
The preterm rabbit pup model with IVH	43
Immunohistochemical staining for cerebellar development.....	46
Histological Analysis of the EGL.....	47
Immunofluorescent labeling for Hb and Hp.....	47
Immunofluorescence for IGF-1 and IGF-1R.....	48
RNA Isolation and Real-Time PCR	48
Quantitative analysis	48
Preterm infants.....	49
Statistics.....	51
Ethical considerations.....	52
Results and comments.....	53
The effect of preterm birth, IVH and caffeine on cerebellar development in preterm rabbit pups.	53
Survival.....	53
Postnatal growth	53
The Insulin-like growth factor system.....	55
External granular layer development.....	57
Cerebellar white matter	59
IVH, cell-free hemoglobin and the cerebellum	60
Retinopathy of prematurity, brain growth and developmental outcome in very preterm infants.....	63
Clinical risk factors for ROP, cerebellar growth and developmental outcome.....	63
Stages of ROP, brain volumes and developmental outcome.....	64
Conclusions.....	67
General discussion and future perspectives	69
Retinopathy of prematurity, brain growth and developmental outcome in very preterm infants.....	69
Cell-free hemoglobin and cerebellar development.....	70
Caffeine, the IGF-1 system and cerebellar development	71
Preterm birth <i>per se</i> and cerebellar development	72
Nutrition and postnatal growth.....	72
External granular layer development.....	73
Svensk sammanfattning	75
Íslensk samantekt	77
Acknowledgements.....	79
References.....	85

List of papers

- I.** **Sveinsdóttir K**, Länsberg JK, Sveinsdóttir S, Garwicz M, Ohlsson L, Hellström A, Smith L, Gram M, Ley D. Impaired cerebellar maturation, growth restriction and circulating Insulin-like Growth Factor 1 in preterm rabbit pups. *Dev Neurosci* 2017;39:487-497
- II.** Agyemang AA, **Sveinsdóttir K**, Vallius S, Sveinsdóttir S, Bruschettini M, Romantsik O, Hellström A, Smith LEH, Ohlsson L, Holmqvist B, Gram M, Ley D. Cerebellar exposure to cell-free hemoglobin following preterm intraventricular hemorrhage: causal in cerebellar damage? *Transl. Stroke Res* 2017;8:461–473
- III.** **Sveinsdóttir K**, Ley D, Hövel H, Fellman V, Hüppi PS, Smith LEH, Hellström A, Hansen-Pupp I. Relationship of retinopathy of prematurity to brain volume at term equivalent age and developmental outcome at 2 years corrected age. *Neonatology* 2018;114:46-52
- IV.** **Sveinsdóttir K**, Bruschettini M, Vallius Kvist S, Gram M, Holmqvist B, Ley D. Preterm birth and caffeine exposure – effects on the IGF-1 system and cerebellar maturation in rabbit pups. *In manuscript*.

Other publications not included in the thesis

Other publications performed within the research team, in which I have participated and am a co-author. These publications are not included in my thesis but have contributed by lending inspiration and extended knowledge and are relevant to paper II as references.

Sveinsdottir S, Gram M, Cinthio M, **Sveinsdóttir K**, Morgelin M, Ley D. Altered expression of aquaporin 1 and 5 in the choroid plexus following preterm intraventricular hemorrhage. *Dev Neurosci* 2014;36:542-51.

Gram M, Sveinsdottir S, Cinthio M, **Sveinsdóttir K**, Hansson S, Mörgelin M, Åkerström B, Ley D. Extracellular hemoglobin - mediator of inflammation and cell death in the choroid plexus following preterm intraventricular hemorrhage. *J Neuroinfl* 2014;11:200.

Ley D, Romantsik O, Vallius S, **Sveinsdóttir K**, Sveinsdottir S, Agyemang AA, Baumgarten M, Mörgelin M, Lutay N, Bruschetti M, Holmqvist B, Gram M. High presence of extracellular hemoglobin in the periventricular white matter following preterm intraventricular hemorrhage. *Front Physiol* 2016;7:330.

Abbreviations

AOP	Apnea of prematurity
BPD	Bronchopulmonary dysplasia
CNS	Central nervous system
CP	Cerebral palsy
CSF	Cerebrospinal fluid
EGL	External granular layer
GA	Gestational age
GW	Gestational week
Hb	Hemoglobin
HI	Hypoxia-ischemia
HIF	Hypoxia inducible factor
HO	Heme oxygenase
Hp	Haptoglobin
IGF-1	Insulin-like growth factor 1
IGFBP	Insulin-like growth factor binding protein
IGF-1R	Insulin-like growth factor receptor
IGL	Internal granular layer
IQR	Interquartile range
IVH	Intraventricular hemorrhage
MDI	Mental developmental index
MRI	Magnetic resonance imaging
NEC	Necrotizing enterocolitis
PDI	Psychomotor developmental index
PHI	Parenchymal hemorrhagic infarction
PHVD	Post-hemorrhagic ventricular dilation
PMA	Postmenstrual age
PVL	Periventricular leukomalacia
ROP	Retinopathy of prematurity
ROS	Reactive oxygen species
SAH	Subarachnoidal hemorrhage
SD	Standard deviation
Shh	Sonic hedgehog
UWMV	Unmyelinated white matter volume
VEGF	Vascular endothelial growth factor
VLBW	Very low birthweight infants

Summary

With improving neonatal intensive care, smaller and extremely preterm infants survive. Although much progress has been made, knowledge on the impact of extreme prematurity and related complications on brain development is still insufficient. The cerebellum is a part of the brain that helps to regulate movements, coordination and cognitive function. During the last trimester of pregnancy the cerebellum is the fastest growing part of the brain and is in a very active proliferative phase before reaching term age which suggests increased vulnerability to environmental changes following very preterm birth. The awareness of the previously underrecognized role of acquired cerebellar abnormalities following preterm birth and their important contribution to neurodevelopmental disability is increasing.

In preterm infants the cerebellar volume as determined by magnetic resonance imaging (MRI) is smaller at term age than in control term infants. Reduced cerebellar volume at term age has been associated with subsequent neurodevelopmental impairment. Cerebellar underdevelopment may ensue from a direct cerebellar injury, such as hemorrhage or infarction, it can be a secondary effect related to damage in the cerebrum such as intraventricular hemorrhage (IVH) but is also shown following preterm birth *per se*, without preceding injury. The mechanisms involved in reduced cerebellar volume following very preterm birth are unknown.

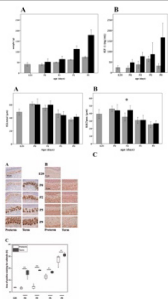
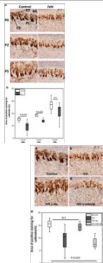
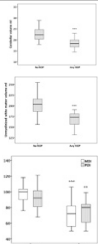
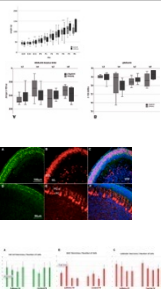
The main question of this thesis is why the cerebellum is smaller in preterm children compared to term born children, and which neonatal events affect or modify cerebellar development? The general hypothesis is that preterm birth *per se* disturbs normal cerebellar development and that certain events following preterm birth, either complications or treatments, can aggravate the underdevelopment.

In the first study we concluded that preterm birth in rabbit pups exhibits a combination of characteristics relevant to human preterm birth and affects cerebellar development compared to term born pups and is appropriate for use in future studies relating to preterm birth and cerebellar development. The second study showed that following IVH in the preterm rabbit pup there is an extensive deposition of cell-free hemoglobin (Hb) in cerebellar cell layers and white matter. The exposure to cell-free Hb was associated with microglial activation, an arrest in neuronal cell proliferation and delayed Purkinje cell maturation. Intraventricular administration of the cell-free Hb scavenger, haptoglobin, partially blocked these effects suggesting that cell-free Hb and its downstream metabolites are causal in cerebellar underdevelopment following IVH. It is well established that the severe form of retinopathy of prematurity (ROP) is associated with impaired

neurodevelopment. ROP is a continuous process of impaired vascular and neuronal retinal development, a neurovascular disease and in the third study on ROP, brain volumes and neurodevelopmental outcome we found that indeed any stage of ROP was associated with reduced cerebellar and unmyelinated white matter volumes at term equivalent age and with lower developmental quotients at 2 years corrected age. We therefore recommend that future studies addressing the association between ROP and development should consider the whole spectrum of ROP. Caffeine is one of the most prescribed drugs in neonatal care and the focus of the fourth study. Experimental data are conflicting, with studies showing either adverse or beneficial effects of caffeine in the developing brain. Enteral caffeine administration to preterm rabbit pups did not reveal any effects on growth or the closely related trophic IGF-1 system in preterm rabbit pups and did not affect key neuronal maturation in the cerebellar external granular layer. It did however increase survival.

To improve the long term outcome for preterm infants a knowledge of damaging factors is crucial to be able to treat, or preferentially prevent adverse events that affect preterm brain maturation. The conclusions of this thesis are that key neuronal maturation is affected following preterm birth in rabbit pups and that IVH aggravates the damage, which can be partially reversed with a cell-free Hb scavenger. The association between retinal and brain development suggests that factors reducing the risk of ROP could also reduce the risk of adverse brain development, including that of cerebellar development. Improved nutritional status and thereby reduced postnatal growth restriction following preterm birth is one example of suggested factors that could reduce the risk of ROP and improve brain growth and neurodevelopment. Caffeine, the widely used medicine in the neonatal care of preterm infants, does not appear to affect cerebellar development and increased survival in the preterm rabbit pups. Future studies will focus on improving postnatal nutritional status and growth following preterm birth in rabbit pups and evaluate longterm behaviour and neurodevelopment. Caffeine could facilitate future studies by increasing the survival in preterm rabbit pups. The other direction should aim at studies on scavenging of cell-free Hb following IVH, *i.e.*, how to get the scavengers into the central nervous system in sufficient levels to reduce cerebral and cerebellar damage following IVH.

The thesis at a glance

	I	II	III	IV
Study question	Does preterm delivery <i>per se</i> in rabbit pups affect serum IGF-1 levels and cause changes in key elements of the developing cerebellum?	How does IVH affect cerebellar development in preterm rabbit pups and is cell-free Hb a causal factor in the process?	How is the relationship between any stage of ROP, brain growth and developmental outcome in preterm infants at 2 years corrected age?	Does caffeine affect the IGF-1 system and key cerebellar cell populations in preterm rabbit pups?
Method	Preterm rabbit pups were compared to term pups by weight, IGF-1 levels and cerebellar proliferation, cell maturation and apoptosis at repeated time points	The cerebellar histology of preterm rabbit pups with IVH were compared to control pups and haptoglobin was used as a Hb scavenger	52 very preterm infants followed and evaluated for grade of ROP, brain volumes by MRI at term age and developmental outcome assessed at 2 y of corrected age using the BSID-II	Preterm rabbit pups treated with caffeine were compared to preterm controls. Serum-IGF-1, hepatic mRNA for IGF-1 and cerebellar IGF-1R distribution and histology were evaluated.
Results	Mean weight and serum IGF-1 were lower in the preterm group at all time-points. Proliferation in the EGL and Purkinje cell morphology was altered in preterm pups. Incidental finding was cerebellar white matter necroses in a subgroup of the preterm pups.	Accumulation of cell-free Hb in cerebellar layers following IVH decreased EGL proliferation and delayed Purkinje cell maturation. The damaging effects were partially reversed by Hb scavenging.	Preterm infants with any stage of ROP had lower cerebellar volumes and unmyelinated white matter volumes and lower mean MDI and PDI at 2 y corrected age as compared to infants without ROP.	Caffeine exposure did not affect weight, serum or hepatic mRNA for IGF-1 at any given time-point. No difference in cerebellar IGF-1R expression, proliferation or Purkinje cell morphology was observed.
Figures				
Conclusion /Take home lessons	The cerebellar development in the preterm rabbit pup model exhibits characteristics relevant to human preterm birth. We propose the model as appropriate for future studies on cerebellar development.	IVH exposes the cerebellum to cell free Hb and causes cerebellar impairment. Scavenging of Hb metabolites may reduce subsequent cerebellar impairment.	Not only severe but all stages of ROP should be considered when evaluating the relationship between ROP, brain development and neurodevelopmental outcome.	Enteral administered caffeine had no clear effects on growth or the closely related trophic IGF-1 system in preterm rabbit pups.

Preface

With improving neonatal intensive care, smaller and extremely preterm infants survive. The increased survival has raised the awareness of the major complications following preterm birth and their negative effect on development of affected infants and in spite of advancing neonatal care, neurodevelopmental complications are common. Fundamental knowledge on the nature of brain injury and brain development is needed to reverse, or preferably prevent the damaging factors. Although much progress has been made, knowledge on the impact of extreme prematurity and related complications on brain development is still insufficient.

Very preterm infants are poorly adjusted to the extrauterine environment. The nutritional changes and insufficient levels of growth factors that follow preterm birth, result in delayed growth and increased risk of multisystem complications. Cerebral insults are well recognized but cerebellar insults are less studied. The awareness of the important contribution of the cerebellum to neurodevelopmental disability is increasing.

This thesis focuses on the cerebellum and its association to common neonatal events following very preterm birth and some of the many unanswered questions. The first paper is a descriptive study that compares some key factors in cerebellar development between preterm and term rabbit pups and evaluates the animal model for future studies. The second paper describes the effect of cerebral IVH on cerebellar development. The third paper presents a clinical study in preterm infants, where the association between retinal development, brain growth including the cerebellum, and later developmental outcome is evaluated. Caffeine is one of the most commonly used drugs in the preterm population and the fourth paper evaluates effects of caffeine on the immature cerebellum.

Background

Very preterm birth

Definitions

Extremely preterm infants are per definition born before 28 completed weeks of gestation (GW) and very preterm infants are born before 32 completed GW. The subject of this thesis applies to infants born extremely and very preterm, together addressed as preterm infants for simplicity unless otherwise specified. Very low birthweight infants are infants with a birthweight $\leq 1500\text{g}$.

Survival

To understand the importance of long-term complications following preterm birth in countries with established modern neonatal care some facts are presented.

In western countries about 1.3-1.5 % of all births are very preterm. In Sweden that equals 1600 infants per year, 60 very preterm infants per year in Iceland and in USA this corresponds to about 65000 very preterm infants born every year according to the national birth registry in each country.

The survival rate is around 90% but complications are common. It is fair to point out that in spite of the high risk of complications the majority of children born preterm have mild or no disabilities at all ¹.

Major complications

The high rates of morbidity following very preterm birth make the limits of viability controversial. The more preterm the higher the risk for complications.

The major complications of preterm birth with the greatest impact on future health are: severe intraventricular hemorrhage (IVH), severe retinopathy of prematurity (ROP), severe bronchopulmonary dysplasia (BPD), periventricular leukomalacia (PVL) and necrotising enterocolitis (NEC).

About 5-10% of VLBW infants develop major motor deficits *i.e.*, cerebral palsy (CP), and 25-50% develop one or more type of cognitive, behavioural, attentional and/or socialization impairment ².

Neurodevelopment in preterm infants

Preterm birth increases the risk of abnormal brain development and developmental problems, even in infants with normal brain ultrasound during their neonatal period ³⁻⁶. Compared to controls, very preterm infants have an increased risk of motor impairment, cognitive impairment, neurobehavioural problems, hearing loss and blindness and these problems persist throughout childhood ³⁻¹⁰.

In the Swedish EXPRESS study of preterm infants born before 27 GW, 30% had moderate to severe cognitive disability, 9.5% had CP, 2% were blind and 2% needed hearing aid. Overall, 14% had severe disability, 20% had moderate, 30% had mild and 36% had no disability at 6.5 years of age ¹. Severe ROP and brain injury independently predicted the risk of death or major disability at 11 years of age ¹¹.

The prevailing brain pathology in preterm infants is diffuse white matter cerebral injury. White matter lesions have primarily been considered to be the origin of neurological impairment and the most contributing neuropathology ¹²⁻¹⁴. Reduced cortical volumes and increased cerebrospinal fluid (CSF) volumes are associated with neurodevelopmental disability both at an early age ¹⁵ as well as at school age ¹⁶ and reduction in white matter volumes of the sensorimotor and midtemporal regions have been linked to adverse mental development at early age ¹⁷. PVL and associated neuronal/axonal damage can be referred to as “encephalopathy of prematurity” ². The increased use of magnetic resonance imaging (MRI) and of the mastoid view on cranial ultrasound imaging ^{18,19} has increased the awareness of the previously underrecognized role of acquired cerebellar abnormalities following preterm birth and their important contribution to the neurodevelopmental disability in this population ²⁰⁻²³. Reduced inferior occipital volumes, including the cerebellum, have been linked to visual ²⁴ and to cognitive impairment ²⁵. Cerebellar damage has been found in as much as 64% of extremely preterm infants who later developed CP ²⁶.

Cerebellum

The role of the cerebellum

The cerebellum is a part of the brain that helps to regulate movements, coordination and cognitive function. The cerebellar role in higher functioning such as cognition, behavior, emotion and language processing, memory and motor learning has become evident in recent years^{23,25,27,28}. A follow up of preterm infants up to adolescence found significant smaller cerebellum on MRI compared to age matched controls and interestingly there was no association between cerebellar volumes and motor impairment but a significant association with several cognitive test scores²⁹. Cerebellar abnormalities have been associated with neuropsychiatric conditions, characterized by perceptual and cognitive disturbances such as autism, attention deficit hyperactivity syndrome (ADHD) and dyslexia^{23,30-33}. The exact mechanisms whereby the cerebellum influences perception is not yet fully understood^{27,34}.

The “cerebellar cognitive affective syndrome” characterized by deficiency in executive functioning, spatial cognition, linguistic abilities and negative changes in personality is a well defined non-motor sequela of cerebellar injury or malformation in older children and adults. It can be applied to preterm born children as well as a developmental form of the syndrome^{23,35}.

Increased recognition of cerebellar involvement in neurodevelopmental impairment following preterm birth emphasizes the importance of understanding the normal events of cerebellar development during the last trimester of pregnancy and how factors associated with preterm birth may interfere with cerebellar maturation.

Cerebellar development

Cerebellar development in humans takes place from the fourth gestational week to 2 years of age. During the last trimester the cerebellum is the fastest growing part of the brain and is in a very active proliferative phase before reaching term age which suggests increased vulnerability to environmental changes following very preterm birth^{36,37}. During this period the cerebellar volume increases 5-fold and the surface area increases 30-fold which makes this period critical in structural and functional cerebellar development²². The adult number of folia is reached at around 2 months postnatal age.

Development of the cerebellar cortex

The mature cerebellum is composed of the cortex, the underlying white matter and the deep nuclei. The cortex is composed of the superficial molecular layer, Purkinje cell layer and the deep granular cell layer. In this thesis the main focus is on the cortex of the developing cerebellum as this is the region with the highest neuronal proliferative rate.

Cerebellar neurons originate in two waves of proliferation. The early wave is from the ventricular zone of the neuroepithelium, takes place at early embryonic stages and gives rise to deep nuclei neurons and all the Purkinje cells. The second wave, from the rhombic lip, takes place during late 2nd and the 3rd trimester of pregnancy in the external granular layer (EGL) where the granule neuron precursors migrate tangentially to cover the surface of the cerebellar plate.

The EGL, is a transient developmental part of the cerebellum where the granule neuron precursors proliferate. This proliferation is regulated by the Purkinje cells that secrete the signalling factor Sonic hedgehog (Shh)^{38,39}. The EGL reaches its peak thickness at around GW 25 and expands horizontally thereafter. As the EGL reaches its peak thickness the post mitotic granule precursor cells start their inward migration to form the internal granular layer (IGL). The EGL disappears at around the 7th postnatal month. The proliferation of granule cell precursors in the EGL and their inward movement to form the IGL is the most important cellular determinant of cerebellar maturation and patterning which makes this period a critical event in cerebellar development²². Granule neurons constitute 95% of all the neurons in the mature cerebellum. Fig 1.

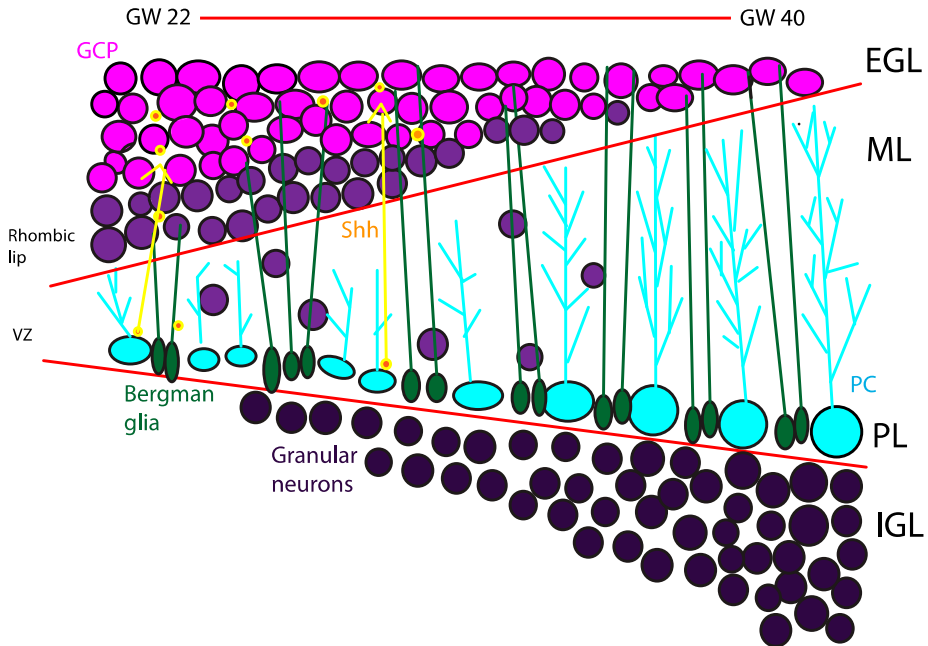


Fig 1. Key factors in cerebellar cortex development.

The granule cell progenitors (light purple) migrate from the rhombic lip and expand transversally throughout the cerebellar surface, become postmitotic (purple) and migrate inward to their permanent place as granule neurons in the IGL (dark purple). Immature Purkinje neurons (turquoise) migrate from the ventricular neuroepithelium to their permanent location in the cerebellar cortex. They secrete the trophic factor Sonic hedgehog (Shh) which stimulates the granule precursor cell proliferation in a paracrine way and their own development in an autocrine way. When in place their dendrites start to grow in the molecular layer towards the EGL and will make important synapses with the granule cell axons. Immature Bergmann glia migrate the same way as Purkinje neurons to their final destination (green) among and inferior to the Purkinje neurons. The Bergman glia send their axons through the forming molecular layer to the EGL to guide the inward movement of the granule cells.

Key factors in cerebellar development

Fig 1.

Granule cells^{22,32,40-42}

Granule cells mature from their precursors in the EGL and after proliferation migrate along the Bergman glia fibers to their permanent position in the IGL/granule cell layer. During their migration through the molecular layer their axons, the parallel fibers, make a critical contact with the Purkinje cell dendrites to establish the cerebellar circuitry. At their final destination in the IGL they are contacted by mossy fiber inputs from the pons.

Purkinje cells^{22,32,40-42}

The Purkinje cells are the key cells of the cerebellar circuit. They migrate to their location in the cerebellum and with time organize themselves into a monolayer, the Purkinje layer. During this time their dendritic tree grows towards the EGL. The Purkinje cells are responsible for the morphogenesis of the cerebellum by secreting Shh that controls the proliferation of granule precursor cells³⁸. The final postsynaptic arrangement of their dendritic tree with the parallel fibers and the mossy fibers is essential for the cerebellar circuit. In return the granule cells have trophic input on the Purkinje cells which is evident when the granule cell population is experimentally compromised which results in poorly aligned Purkinje cells and morphologically altered dendrites. The Purkinje cells receive all input to the cerebellar cortex via direct contact with climbing fibers from inferior olivary nuclei and indirect, via granule cells, from mossy fibers from the nuclei of the spinal cord, brainstem and deep cerebellar nuclei. Their axons are the sole output from the cerebellar cortex to the thalamus and brain stem.

Bergman glia^{22,32,40-42}

Bergman glia are astrocytes that direct neuronal migration and guide the critical contact between the granule neurons and the Purkinje neurons and the formation of the IGL. They reside immediately around/below the Purkinje cell layer and their axons extend through the molecular layer towards the EGL. Bergman glia have a repair mechanism that allows them to switch from a differentiated to proliferative state following cerebellar injury and Purkinje cell destruction. Shh from the Purkinje cells controls the differentiation of Bergman glia⁴³.

Sonic hedgehog - Shh

Sonic hedgehog (Shh) is central in the growth and patterning of the cerebellum³⁷. As previously mentioned Shh is essential for proliferation in the EGL and for Bergman glia differentiation and thereby formation of the molecular layer and the EGL^{38,44}. By controlling the proliferation in the EGL it controls the extent of cerebellar foliation⁴⁵. The Purkinje cells also secrete Shh in an autocrine way for their own development³⁷. In the cerebellar white matter Shh controls oligodendrocyte proliferation and differentiation and thereby myelination⁴⁶.

Blocking Shh signalling *in vivo* leads to a hypoplastic cerebellum with abnormal foliation. The differentiated granule neurons and the Bergman glia are fewer or absent and the Purkinje cells are abnormally positioned³⁷. On the other hand excessive Shh signalling leads to uncontrolled proliferation of granule cell precursors which is thought to be the underlying defect in medulloblastoma, a pediatric brain tumor of the cerebellum⁴⁴.

IGF-1

Insulin-like growth factor 1 (IGF-1) is a trophic, anabolic and neuroprotective growth factor that plays an essential role in the proliferation of both the Purkinje cell layer and the EGL and promotes neuronal survival by blocking apoptosis²². IGF-1 has a supporting roll in Shh-induced proliferation of EGL which is clearly shown by blocking the IGF-1 receptor (IGF-1R) which highly decreases granule precursor cell proliferation⁴⁷.

The effect of preterm birth on cerebellar development

The rapid growth of the cerebellum from GW 20 to 40 (from 1cm³ to 25 cm³) renders the cerebellum very sensitive to injury^{36,48,49}.

In preterm infants the cerebellar volume as determined by MRI is smaller at term age than in control term infants^{36,50,51}. Cerebellar underdevelopment may ensue either from a direct cerebellar injury, such as hemorrhage or infarction, with a secondary effect related to damage in the cerebrum at a remote but neurally connected area of the brain (diaschisis)^{48,52,53}. Cerebellar underdevelopment is also shown following preterm birth *per se*, without preceding injury^{6,36,54}.

Reduced cerebellar volume at term age has been associated with subsequent neurodevelopmental impairment^{6,25,53-56}. The mechanisms involved in reduced cerebellar volume following very preterm birth are unknown.

Incidence of cerebellar injuries in preterm infants

The incidence ranges from about 2-20% in different studies with different study subjects. Up to 1 in 5 of preterm infants have been shown to have visible cerebellar injury on MRI with the highest rate in the smallest and most immature infants. On ultrasound, 2-15% of preterm infants had cerebellar insults, again with the highest incidence in the smallest infants^{19,57}.

Prevalence of cerebellar injury is described in up to 64% of infants with CP following IVH and preterm birth^{22,58}.

Direct cerebellar injury

Of preterm infants with cerebellar lesions, which are mainly ischemic or hemorrhagic lesions, 23% had isolated cerebellar injury and the remaining infants (77%) had cerebral lesions as well. Most of the cerebellar injuries are hemispheric (71%)⁵⁷. A study of 112 preterm infants found cerebellar lesions in 15%, more common in infants with moderate/severe IVH (4/17 infants vs 7/95)²⁵. Direct cerebellar injury is associated with high risk (66%) for neurological abnormalities. Hypotonia, gait abnormalities, extraocular disturbances and language impairments

have been associated with hemorrhage in the cerebellar hemisphere while socialization, behavioural deficits and autism spectrum have been associated with vermian involvement²².

Isolated cerebellar insults are usually followed by cerebellar atrophy and associated with disability in motor, language and cognitive functions³⁰.

Preterm infants with smaller cerebellar hemorrhage (punctate haemorrhage) only detected on MRI during the stay in the neonatal ward and at term equivalent age, had a statistically worse motor outcome at 5 years of age but no difference in cognitive impairment compared to infants without cerebellar hemorrhage⁵⁹.

Indirect cerebellar injuries

Cerebellar hypoplasia has repeatedly been shown to be associated with supratentorial IVH in preterm infants and to be a potential component in neurological disability^{25,49,60,61}. The severity of IVH has been shown to be inversely correlated with cerebellar volume⁴⁸. Circulating blood products in the cerebrospinal fluid (CSF) following IVH is one of possible mechanisms for the indirect cerebellar injury since cerebellar volume reduction has been shown to be equally associated with ipsilateral and contralateral IVH⁴⁸. The main target in the cerebellum would hypothetically be the granule precursor cells in the EGL that is in direct interphase with the subarachnoidal space. MRI at term age shows infratentorial hemosiderin deposits on the cerebellar surface in the posterior fossa in up to 80% of preterm infants with IVH and disrupted cerebellar development without any sign of primary cerebellar hemorrhage⁵⁰.

The diaschisis effect works in both ways with impaired trophic interactions between the cerebellum and cerebrum. Cerebral injury is associated with volume reduction of the contralateral cerebellar hemisphere and isolated cerebellar injury has an effect on remote growth and volumetric development of the contralateral cerebral hemisphere in regions known to be activated by the afferent pathways from the contralateral cerebellum^{52,62}. This remote effect of secondary underdevelopment of the cerebral cortical projection regions is highly correlated with developmental impairment. Reduced volume of the dorsolateral prefrontal cortex is highly correlated to early signs of autism, to reduced volume of the sensorimotor cortex and to adverse gross motor scores, with premotor and mid-temporal cortical volumes correlating to expressive language impairment³¹.

The motor sequelae following cerebellar injury in preterm infants are hypotonia and delayed motor development as compared to the characteristic symptoms of nystagmus, ataxia and intention tremor seen after injury in the adult cerebellum³⁰.

Cerebellar underdevelopment following preterm birth per se

Cerebellar underdevelopment may represent the most common type of cerebellar abnormality in very preterm infants. The underlying causes are likely multifactorial including lower gestational age, total brain volume, illness severity and postnatal growth parameters³⁶. A histological study showed that preterm birth disrupts the developmental program of the cerebellum, by affecting specific cell types rather than causing generalized changes⁶³.

Preterm birth leads to abrupt nutritional changes following the loss of placental support and trophic deprivation due to less than optimal postnatal nutrition⁶⁴. Postnatal growth retardation commonly follows preterm birth⁶⁵ and has been associated with decreased brain volumes and with impaired neurodevelopmental outcome⁶⁶. Cerebellar volumes have been shown to be significantly related to head circumference and weight at term equivalent age³⁶. Induced intrauterine growth restriction resulted in volume reduction of the molecular layer, IGL and white matter of the cerebellum as well as reduced number of Purkinje cells in guinea pigs⁶⁷. Placental insufficiency has been shown to alter brain development and the cerebellar white matter in humans⁶⁸.

Hypoxic events are relatively common in preterm infants due to respiratory and cardiovascular instability. Preterm infants also have a high risk of infections and inflammation. Experimental studies have shown that both hypoxia-ischemia (HI) and inflammation negatively affect the granule cell proliferation and Purkinje cells in the cerebellum⁶⁹. In rats, hypoxia alone was more damaging to the neuronal population of the cerebellum than hypoxia-ischemia⁷⁰.

Following preterm birth there is an abrupt change from the hypoxic environment *in utero* to the relative hyperoxic environment *ex utero*. Impaired granule cell development and Purkinje cell functioning was shown after a period of hyperoxia in rats which could indicate postnatal oxygen toxicity as a factor associated with cerebellar maldevelopment⁷¹.

Preterm infants are exposed to high levels of glucocorticoids, either from an exogenous source as in treatment or intrinsically due to high stress levels. The EGL in the cerebellum has the highest number of glucocorticoid receptors in the brain^{72,73}. Postnatal, but not antenatal steroid exposure has been associated with impaired cerebellar growth⁷⁴. Chronic treatment with glucocorticoids in mouse pups inhibited Shh-induced proliferation of granule precursor cells *in vitro* whereas acute treatment caused transient apoptosis⁷⁵.

Another possible mechanism for disrupted cerebellar development is loss of trophic factors such as IGF-1 with its proliferating and differentiating effects in the cerebellum⁷⁶. Levels of circulating IGF-1 are low following preterm birth⁵⁶ and

low levels of circulating IGF-1 are associated with decreased brain volumes at term age, with the cerebellum exhibiting the strongest correlation⁵¹.

In summary: different theories have been postulated addressing cerebellar underdevelopment following preterm birth but the neuropathological basis is as yet largely unknown.

Cerebral intraventricular hemorrhage - IVH

Cerebral intraventricular hemorrhage (IVH) continues to be a major complication of preterm birth following increasing survival of very immature infants⁷⁷. Severe IVH occurs in about 15-20% of preterm infants^{78,79}. Most hemorrhages occur in the first postnatal week, usually during the first 3 postnatal days and the incidence increases with decreasing gestational age⁸⁰.

Pathophysiology

IVH initiates in the periventricular germinal matrix of the lateral ventricles between the ventricular wall and the head of the caudate nucleus. The germinal matrix consists of rapidly dividing neuronal and glial progenitor cells and of fragile vessels which makes the area highly vulnerable to hemorrhage^{81,82}. The germinal matrix reaches a maximal thickness around GW 24 and thereafter reduces in size and has disappeared around GW 35 which is why IVH is predominantly a disease of preterm infants⁸³.

The etiology is multifactorial and complex. An inherent fragility of the immature germinal matrix vasculature is thought to set the ground for the hemorrhage⁸¹. The germinal matrix is exposed to insults of arterial ischemia-reperfusion and venous congestion because it is situated within an arterial end zone and connected to the deep galenic venous system⁸⁴. Therefore, anything that causes disturbance in cerebral blood flow in the preterm infant can induce IVH⁸¹.

Diagnosis and classification

Because of the high incidence of IVH most modern neonatal intensive care units have screening programs for IVH. The diagnosis is easily made by bedside ultrasound and so is the follow up of existing hemorrhages.

The degree of IVH is based on the extent of the bleeding and the size of the ventricles⁸⁵. Grade I: confined within the subependymal germinal matrix. Grade II: bleeding in the germinal matrix that extends in to the ventricles but comprises less than 50% of the volume. These are considered as mild IVH. Grade III: has more extensive intraventricular part that comprises more than 50% of the volume

and causes ventricular distension. Grade IV or rather parenchymal hemorrhagic infarction (PHI) is an IVH with parenchymal involvement. Grade III and PHI are considered as severe IVH.

Complications following IVH

The mortality of infants with severe IVH is 20-50% in the neonatal period. Approximately 40-80% of preterm infants surviving severe IVH develop posthemorrhagic hydrocephalus (PHVD) and/or neurodevelopmental impairment such as intellectual impairment or CP ^{79,81,86-88}.

The long term-effects of mild IVH are not as clear with studies showing either no association to long-term complications ⁸⁹ or association to neurodevelopmental impairment such as neurosensory impairment ⁹⁰⁻⁹².

Prevention of IVH remains an unsolved issue despite major efforts to elucidate the pathogenesis. To date there is no treatment available to prevent infants from developing serious neurological impairment following IVH.

IVH and the cerebellum

As previously noted, cerebellar hypoplasia has repeatedly been shown to be associated with supratentorial IVH in preterm infants. Following IVH 80% of preterm infants had hemosiderin deposits on the cerebellar surface without a primary cerebellar hemorrhage as a cause ⁵⁰. Hemosiderin has been associated with cerebellar atrophy in children with severe head injury and adults with cerebellar siderosis ^{93,94}. The hypothesis that hemosiderin deposits may be causal in cerebellar hypoplasia following IVH is supported by the high incidence of olivo-ponto-cerebellar pathologies and increased glial reaction in preterm infants with subarachnoidal hemorrhage (SAH) ^{95,96}.

The damaging effect of cell-free hemoglobin in the brain

IVH grade II or more leads to deposit of extravasated blood into the CSF and the subsequent hemolysis causes high levels of cell-free hemoglobin (Hb) in the CSF. Fig 2.

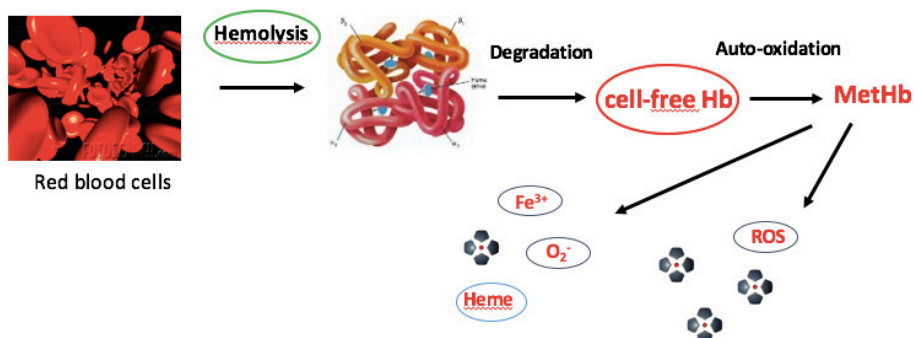


Fig 2. Degradation of hemoglobin.

Following hemolysis of red blood cells there is a rapid degradation of the red blood cells releasing cell-free hemoglobin (Hb). The cell-free hemoglobin is auto-oxidized to the toxic methemoglobin (MetHb) which further degrades releasing free iron (Fe^{3+}), radical oxygen species (ROS), heme and superoxid (O_2^-), all of which are toxic to the immature brain. Figure used with permission from Sveinsdottir S.

Cell-free Hb and its metabolites free heme, iron, reactive oxygen species (ROS) and free radicals can be highly damaging to cells, lipids, proteins and DNA through oxidative modification, fragmentation and cross linking causing damage in cells and tissues⁹⁷⁻¹⁰⁰. They have been shown to induce cytotoxic, oxidative and inflammatory pathways in the CSF and choroid plexus ependyma leading to tissue damage and cell death following preterm rabbit pup IVH¹⁰¹⁻¹⁰³. High accumulation of cell-free Hb in periventricular white matter was observed following IVH in preterm rabbit pups¹⁰⁴.

The neurotoxicity of cell-free Hb and its metabolites has been reported after intraventricular, intraparenchymal and subarachnoid hemorrhage¹⁰²⁻¹⁰⁹. Preterm infants with PHVD have high levels of free iron in the CSF for weeks after an IVH because of the limited ability of the central nervous system (CNS) to discharge iron¹¹⁰.

Hemoglobin clearance – the haptoglobin system

The haptoglobin system (Hp) is the main endogenous scavenging system for cell-free Hb. Cell-free Hb binds to Hp forming an inert Hb-Hp complex which channels the Hb molecule for internalization and degradation by CD-163 positive macrophages^{111,112}.

The importance of the Hp-scavenger system in the human brain has been scarcely studied, particularly in the immature brain. The levels of endogenous Hp in the human brain are very low with a intrathecal Hb-Hp complex clearance capacity that is 50000 fold lower than in the circulation. The levels of Hp in the systemic

circulation of very preterm infants has been reported to be very low ¹¹³. Since systemic Hp contributes to intrathecal Hp the intrathecal levels of endogenous Hp is expected to be extremely low in this population and would therefore quickly be saturated with a residual inability to inactivate cell-free Hb following an episode of SAH or an IVH ¹¹⁴. Therefore very preterm infants are expected to be highly vulnerable to cell-free Hb.

Retinopathy of prematurity

Preterm birth coincides with a critical period of brain and vascular development ²². The retina is a part of the CNS and is a complex and highly vascularized neural tissue that is incompletely developed at very preterm birth. Retarded retinal vascular growth can lead to the development of retinopathy of prematurity, ROP, which is a potentially blinding disease associated with visual and/or neurodevelopmental impairment ¹¹⁵⁻¹¹⁸. Presence of ROP is closely associated with the presence of other postnatal complications, such as poor postnatal growth, hyperglykemia, septicaemia, lung disease, NEC and IVH ¹¹⁹. Preterm infants who develop severe ROP are at increased risk of visual ¹¹⁵ and non visual neurodevelopmental comorbidities and of delayed white matter maturation estimated by MRI at early postnatal age and at term equivalent age ^{116,120-122}. These findings suggest presence of common mechanisms in the development of these complications ¹²³ and support the concept of the retina being referred to as “the mirror of the brain” ¹²⁴.

Incidence

Incidence of ROP differs according to regions and countries and the comparisons of the incidence of ROP from population-based studies are difficult because of different study designs, study subjects and different neonatal strategies between units. According to the Swedish registry for ROP (SWEDROP), analysis of ROP incidence from 2008-2015 showed that 32% of all infants born before GW 31 had ROP and 5.7% of the infants were treated for ROP ¹²⁵.

Retinal development in utero

In humans the retinal vascularization begins during the 4th month of gestation, starting centrally from the optic nerve and reaches the periphery right before birth. Therefore the retina of infants born very premature is incompletely vascularized and has a peripheral avascular zone ¹¹⁸. *In utero* the vascular and the anteriorly located neural retinal development are intertwined. Increased oxygen demand from the neural retinal development leads to a relative hypoxia that stimulates the expression of hypoxia-induced vasoproliferative factor (VEGF) to induce normal

vessel development. Normally, as new vessels form the hypoxia decreases and VEGF expression is reduced via a local feedback mechanism. This normal feedback mechanism is disturbed by preterm birth.

Pathophysiology

ROP is a biphasic disease consisting of an initial phase of vessel loss from birth to a postmenstrual age (PMA) of 30-32 weeks followed by a second phase of excessive vessel proliferation, from a PMA of 32-34 weeks and onward until the retina is fully vascularized. The first phase of ROP occurs with the change from a relative hypoxic environment *in utero* to a postnatal hyperoxic environment which causes suppression of VEGF and erythropoietin and an arrest of vessel formation and eventually regression of existing vessels. With increasing metabolic demands, hypoxia will develop in the poorly vascularized retina which stimulates VEGF over-expression and the abnormal vessel formation that defines the second phase of ROP. New vessels form at the junction of the vascular and avascular zone and in the preterm infant this can become pathological with scar tissue formation and retinal detachment^{117,118}.

ROP is divided into 5 different stages according to location and degree of vessel abnormality. Stage 1 and 2 are considered mild and stage 3-5 severe¹²⁶.

The retina is considered to be a part of the CNS and there is a strong association between severe ROP and neurodevelopmental and/or visual impairment^{117,118}.

Risk factors

Low birth weight and gestational age.

The lower the weight and gestational age the higher the risk of developing ROP^{116,127}. The lower the gestational age the longer is the phase 1 of ROP as it lasts until around PMA 30 weeks. A large multinational study found birth weight SD score (SDS) to be a major risk factor for developing ROP requiring treatment but the risk was related to degree of immaturity and only present in more mature infants ≥ 26 weeks¹²⁸. Similar findings are shown by Lee et al¹²⁹.

Low serum IGF-1 levels

IGF-1 acts indirectly as a permissive factor in retinal development by controlling the maximal function of VEGF stimulation on vessel growth^{118,130}. Lack of IGF-1 in mice prevents normal retinal vascular growth despite the presence of vascular VEGF¹³⁰. In preterm infants, low circulatory IGF-1 concentrations are associated with development of severe ROP, where the duration and degree of low IGF-1 levels corresponds to the severity of ROP¹¹⁹. Furthermore, decreased postnatal IGF-1 levels as compared to corresponding levels *in utero* have been associated

with impaired postnatal growth and with decreased brain volumes at term equivalent age in preterm infants^{51,64}.

ROP, postnatal growth retardation and nutritional intake

Recent evidence points to poor weight gain during the first weeks of life in addition to low gestational age (GA) as being the strongest predictor of ROP. Poor early postnatal weight gain during the first phase of ROP is related to development of severe ROP and low brain volumes at term equivalent age^{123,128,131}.

A retardation of headgrowth which can be considered a proxy of brain growth occurs in almost all preterm infants after birth which coincides with the initial suppression of retinal vascular growth. If the head circumference in the preterm infants is 2,5 SD or more below the mean at PMA of 30 weeks, the risk of developing severe ROP is five-fold compared to infants with less head growth retardation¹²³. This suggests common mechanisms which influence growth in both the retina and the brain.

An optimal nutritional intake in very preterm infants is usually difficult to achieve. Low energy intake (fat and carbohydrates) during the first 4 weeks of life in extremely preterm infants was an independent risk factor for developing severe ROP¹³². Recently low postnatal levels of arachidonic acid have been shown as a predictive factor for development of ROP¹³³.

Oxygen treatment

Supplemental oxygen in preterm infants causing changes in circulating oxygen tensions interferes with the VEGF driven angiogenesis. Hyperoxia suppresses VEGF expression leading to regression of existing vessels and cessation of vessel growth. Lower oxygen targets are associated with reduced ROP but increased mortality^{134,135}. Theoretically oxygen target should be lower during phase 1 of ROP and higher during the later phase. A meta-analysis showed the same results; lower oxygen targets in the first weeks of life reduced the risk for ROP and higher oxygen targets after GW 32 slowed the progress of ROP¹³⁶. The optimal oxygen target to increase survival and optimize organ growth without increasing the incidence of ROP is not known. Improved control and avoidance of fluctuations is recommended.

ROP and long-term complications

Ophthalmologic outcome/visual impairment

Ophthalmologic problems are common in preterm infants. At 6.5 year follow up of the Swedish EXPRESS cohort of infants born before 27 GW, ophthalmologic abnormality was diagnosed in 38% of the preterm children compared to 6% of

term controls. Treatment requiring ROP was a stronger risk factor than GA¹¹⁵ for visual impairment. Contributing factors for the high incidence of ophthalmologic problems at 6.5 years of age were prematurity *per se*, treatment requiring ROP, severe BPD and CP¹³⁷. Vision is related to white matter development in the optic radiation at term equivalent age¹³⁸. In adolescents born preterm, impaired vision was associated with altered optic radiation structure¹³⁹. Impaired visuomotor control, such as smooth pursuit and strabismus, at 2 years corrected age is related to smaller inferior occipital volumes, including the cerebellum, at term corrected age in preterm infants²⁴.

Neurodevelopmental impairment

Preterm infants with affected visual status also have worse neurodevelopmental outcome^{140,141}.

Some but not all of the associations between ROP and encephalopathy of prematurity can be explained by common risk factors. After adjusting for common risk factors, infants with ROP were more likely to have lower developmental quotients at 2 years corrected age compared to infants without ROP and the more severe the ROP, the lower the quotients¹¹⁶. In the CryoROP multicenter study cohort, severity of neonatal ROP was found to be a marker for functional disability at 5.5 years of age. The more severe the ROP, the fewer the children who were globally functionally normal¹⁴¹ and those with unfavorable vision did even worse¹⁴⁰. This shows that despite favorable visual outcome, severe ROP strongly predicts non-visual disability, even without preceding brain injury.

Assessment with spectral-domain optical coherence tomography of retinal nerve fiber layer thickness in preterm born children showed reduced thickness compared to term born children. Retinal nerve fiber layer thickness correlated with ROP stage¹⁴². The optic nerve can be considered to be “the window in to the brain”. With diffusion tensor imaging technique, treatment-requiring ROP predicted white matter maturational delay in the visual and motor pathways at term equivalent age. The delayed white matter maturation was present independently of other signs of brain injury and was associated with poorer motor and cognitive outcome at 18 months¹²².

A recent case-control study comparing severe ROP with no or stage 1 ROP found severe ROP to be associated with reduced cerebellar and brainstem volumes at term age and with neurodevelopmental deficits at 2 years of age¹⁴³.

In summary: preterm infants who develop severe ROP are, besides ophthalmological disabilities, more likely to have long-term evidence of brain damage including CP, developmental delay, lower scores on verbal and performance tests and impaired conceptual and spatial ability compared with their

peers without severe ROP. Common risk factors account for some of the associations but not all. Factors reducing the risk of ROP could also reduce the risk of adverse brain development.

Caffeine

Caffeine is one of the most prescribed drugs in the neonatal care of preterm infants¹⁴⁴. It is standard of care to treat the common problem of apnea of prematurity (AOP) with caffeine thereby aiming to reduce supportive ventilator treatment¹⁴⁵. Considering that caffeine has been used in neonatal care for over 40 years there are surprisingly few case-control studies and follow-up studies evaluating caffeine treatment in the preterm infants.

Caffeine is a non-selective antagonist of adenosine A₁ and A_{2A} receptors and increases neuronal respiratory activity and oxygen consumption. Its hydrophobic property allows it to easily enter the CNS via the blood brain barrier¹⁴⁶.

There have been concerns regarding adverse effects of caffeine in the developing brain^{147,148}. Adenosine has numerous regulating functions in the mature brain such as sleep regulation and arousal via neuronal excitability and autoregulation of cerebral blood flow according to energy demand¹⁴⁹. Adenosine has been shown to be neuroprotective in the mature brain by decreasing ischemic brain injury via the A₁ receptor and antagonists, like caffeine, to have the opposite effect¹⁵⁰. However the adenosine function in the immature brain seems to be different^{147,151,152}.

Clinical data, effects of caffeine on the preterm brain

In the largest clinical study on caffeine and preterm infants, the CAP trial, caffeine was given to preterm infants within 10 days from birth with a loading dose of 20 mg/kg and a maintenance dose of 5-10 mg/kg/day. Caffeine reduced the risk for BPD, shortened the time of assisted ventilation and reduced the need for postnatal steroid treatment¹⁵³. At 18 months, survival without neurodevelopmental delay (cerebral palsy and cognitive delay) was increased in treated infants¹⁵⁴ but at 5 year follow-up that difference was no longer significant even though the caffeine treated infants had better motor function and better visual development. An MRI substudy showed improved white matter development and more mature organization of the white matter in the caffeine group at 5 years of age¹⁵³⁻¹⁵⁵. The results of the CAP trial indicated that caffeine treatment could have a neuroprotective effect on the developing preterm brain. Few clinical studies have shown adverse effects of caffeine treatment except for a temporary negative effect on weight gain that might either be associated with the diuretic effect of caffeine

¹⁵⁶ or the increasing oxygen consumption and increased energy expenditure following caffeine treatment ¹⁵⁷.

Experimental data, effects of caffeine on the preterm brain

Experimental data on the effect of caffeine on brain development are conflicting showing either beneficial or adverse effects. In the context of HI the interaction of adenosine with the adenosine A_{2A} receptor appears clearly neuro-protective ¹⁵². In mice, a single dose of caffeine 5 mg/kg administered directly after HI reduced brain atrophy by 44% and improved behavior compared to controls ¹⁵⁸. Rat pups exposed to caffeine during the first week of life and then exposed to HI exhibited reduced brain damage by 30% compared to controls even at a low dose of caffeine ¹⁵⁹. Experimental studies have shown increased arborisation of the pyramidal neurons of the prefrontal cortex and another study decreased astrocytogenesis in cerebral white matter following caffeine exposure ¹⁴⁷.

Effects of caffeine on the developing cerebellum

Studies on how caffeine affects the cerebellum are scarce. A caffeine-supplemented diet fed to newborn rats increased RNA and protein content within the cerebellum but had the opposite effect on the brain ¹⁶⁰. Caffeine exposure through maternal milk during the first 10 days of life in rat pups resulted in significantly smaller cerebellum and an increase in saturated fatty acids in the cerebellum ¹⁶¹. Maternal caffeine consumption during gestation and lactation had beneficial effect on the developing cerebellum in rat pups exposed to febrile seizures. It reduced oxygen stress and apoptotic signaling and had a positive effect on fine motor coordination and gait disturbances at long term follow up ¹⁶².

A pilot study in preterm infants receiving 4 times the currently recommended loading dose of caffeine showed a significant increase in cerebellar hemorrhage ¹⁶³. Early exposure to caffeine has been shown to decrease the density of the adenosine A₁ receptor in the cerebellar molecular layer at young adult age ¹⁶⁴.

Caffeine and ROP

In the CAP trial the incidence of severe ROP was reduced in caffeine treated infants ¹⁵³. The multifactorial pathogenesis of ROP suggests that multifactorial treatment might be needed to address all the pathogenic factors: growth factors, oxidative stress, inflammatory response and membrane disruption. Treatment with a combination of caffeine (reduces oxidative stress) and Ketolorac (anti-inflammatory effect) in newborn rats with ROP significantly reduced the occurrence of oxygen-induced retinopathy ¹⁶⁵.

The Insulin-like growth factor system

IGF-1 is an anabolic and neuroprotective hormone with proliferative, differentiating, anti-apoptotic and metabolic effects essential for fetal growth and pre- and postnatal brain development^{166,167}. The developing brain has an enormous metabolic need in the early postnatal period. IGF-1 controls the glucose metabolism in the developing brain in a similar way as to how insulin controls the peripheral glucose utilization¹⁶⁸. IGF-1 is primarily produced and released into the circulation from the liver or synthesized locally, for example in neuronal cells in the brain and retina. IGF-1 mediates most of its effects through its main receptor IGF-1R, located on the surface of different cell types in all tissues. IGF-1 is bound to its binding proteins IGFBP that regulate the availability of IGF-1. Transfer of circulating IGF-1 from the circulation to the immature brain is predominantly via the CSF¹⁶⁹, where IGF-1 uses transport mechanisms in the choroid plexus to enter the brain¹⁷⁰.

IGF-1 plays many roles in different organs but in this thesis the focus is on its role in parts of the CNS, *i.e.*, the cerebellum and retina, as well as its interaction with caffeine in the very preterm infant.

IGF-1 in utero

IGF-1 secreted from the placenta is the dominant regulator of fetal growth and the fetal levels are regulated by a feedback loop between the placenta and the fetus depending on the fetal levels of IGF-1. The placental transfer of IGF-1 is mediated by insulin via direct or indirect effects of nutrients from the mother¹⁷¹⁻¹⁷⁴. IGF-1 concentrations increase through gestation with a rapid increase in 3rd trimester with accelerating growth of the fetus¹⁶⁶. In humans, inability to produce IGF-1 results in severe intrauterine growth restriction and a reduction in neuronal populations¹⁷⁵.

IGF-1 and preterm birth

After very preterm birth there is a significant decrease in levels of circulating IGF-1 following interruption of the feto-placental unit. The IGF-1 levels remain very low after birth because of the preterm infants inability to maintain the corresponding intrauterine levels. Common conditions such as starvation, infections and stress can further reduce the IGF-1 levels¹¹⁷. Full term infants in comparison regain normal levels within a few days after birth. IGF-1 is related to nutritional supply and is essential for both pre- and postnatal growth as well as for growth of the retina and the brain. Preterm infants appear to have deficient nutrient utilisation as nutrient intake is not associated with early postnatal growth or increased serum IGF-1 as it is in children and adults⁶⁴. Variations in protein and

caloric intake have limited influence on circulating IGF-1 levels during growth restriction but a significant influence during established catch-up growth⁶⁴.

Very preterm birth is followed by a period of growth restriction and with low levels of IGF-1 that do not respond to improved nutritional intake alone. In rats fed half of the required calories, exogenous IGF-1 increased weight during the state of undernutrition¹⁷⁶. A clear association between IGF-1 and nutritional intake and accelerated growth, is first seen from around PMA of 30 weeks with the initiation of catch-up growth and normal levels are attained at around 1 month corrected age^{64,119}. This supports that both optimized nutrition and higher levels of IGF-1 are needed for optimal postnatal growth in preterm infants.

Besides generalized growth retardation including reduced head circumference¹²³ the decrease in serum IGF-1 levels after preterm birth is associated with multiple major morbidities such as lower brain volumes (unmyelinated white matter, gray matter and cerebellar volume) at term age, with strongest correlation to cerebellar volume⁵⁶, BPD, ROP¹⁷⁷, IVH and impaired neurodevelopmental outcome at 2 years corrected age^{51,56,66,119}.

IGF-1 and cerebellar development,

IGF-1 and its receptor are highly expressed and have coordinated interactions in the cerebellum during development with the highest expression in the EGL^{39,178,179}. IGF-1 expression is activated in the cerebellum just before birth in a subset of Purkinje cells. IGF-1 influences all of the mechanism in normal brain development, apart from migration, *i.e.*, proliferation, differentiation, maturation and apoptosis¹⁸⁰⁻¹⁸². IGF-1 affects the development of the majority of cell populations in the cerebellum, at least for a limited time¹⁷⁸. 0-mutant IGF-1 mice show an overall drop in cell number in the cerebellum which results in a reduced but yet normal development¹⁸³. IGF-1 has a supporting role in Shh-induced proliferation of the cerebellar granule neurons which sets the ground for future cerebellar development⁴⁷. Besides its role in the proliferation it has anti-apoptotic effect on the granule cells¹⁸⁴. IGF-1 supports the survival of Bergman glia precursor and is critical for their normal development¹⁸³. Overexpression of IGF-1 in transgenic mice and cell cultures establishes its role as a trophic factor by increasing cerebellar growth (both weight, DNA, RNA and protein content), induces a remarkable increase in the number of granule cells by stimulating their proliferation and increases the number of Purkinje cells by promoting their survival. This increase in cell number is followed by increased thickness of the molecular layer. In the cerebellar white matter IGF-1 stimulates oligodendrocytes proliferation and thereby increases myelination. These stimulating effects of IGF-1 occur although IGF-1 concentrations in the brain are the lowest of any tissue measured, indicating that brain growth is extremely sensitive to IGF-1 levels^{39,185}.

The neuroprotective effects of IGF-1 is principally by suppression of intrinsic death signalling cascade¹⁸⁶

The role of IGF-1 in synaptogenesis has been shown in the brain¹⁸² and applies to the cerebellum as well where IGF-1 has a promoting role in synaptogenesis between the Purkinje cells and neurons outside the cerebellum and in the climbing fibers from the inferior olivary nucleus¹⁸⁷.

IGF-1 and ROP

IGF-1 is a critical non-oxygen-regulated factor necessary for normal retinal neurovascular development and stabilises newly formed vessels¹⁸⁸. Lack of IGF-1 in knockout mice prevents normal retinal vascular growth¹³⁰. Low serum IGF-1 levels are associated with a 2.2-fold increased risk of ROP in very preterm infants¹¹⁹ and there is a strong association between duration of low IGF-1 and severity of ROP¹¹⁹. The most immature infants have the longest duration of low IGF-1 and longest time of phase 1 of ROP and are therefore at highest risk for developing severe ROP.

The IGF system and caffeine

Downregulation of IGF-1 and the IGF-1R in association with caffeine exposure has been shown in different settings. Maternal prenatal caffeine consumption can inhibit skeletal growth by increasing fetal exposure to maternal glucocorticoids which lowers IGF-1 signaling pathway activity, decreasing IGF-1 mRNA expression levels in the liver and growth plate¹⁸⁹. Fetal rats exposed to prenatal caffeine also showed glucocorticoid stimulation in liver and reduced expression of IGF-1 and IGF-1 receptors³⁴. Downregulation of IGF-1R has been shown with increased caffeine consumption in breast cancer patients¹⁹⁰. There is a paucity of knowledge on how caffeine treatment interacts with the endogenous IGF system following preterm birth.

Developmental similarities in the retina and cerebellum

Features of retinal and brain development have links to common pathways that may in turn be affected by insults associated with very preterm birth. The antecedent events that affect migration and proliferation may be equally important in both the retina and brain. This idea is supported by similar risk factors associated with ROP, lower brain volumes and impaired neurodevelopment.

The Sonic hedgehog is a key factor in both cerebellar and retinal proliferation during early development. Shh is secreted by Purkinje neurons and stimulates proliferation of granule precursor cells in the cerebellum. Similarly, when Shh is

secreted by retinal ganglion cells it stimulates retinal progenitor cells to cell-fate determination, proliferation, and self renewal^{44,191}.

Expression patterns of components of the IGF system appear to be coordinated in the cerebellum and retina during development; for example, IGF-1 mRNA is expressed in Purkinje cell bodies in the cerebellum and in retinal ganglion cells and the IGF-1R receptor mRNA expressed in glial cells, that is the Bergman glia in the cerebellum and the Müller cells in the retina¹⁷⁸.

Periventricular leukomalacia induced by ischemia is associated with concurrent retinal vascular damage in rat pups¹⁹². Hypoxia-inducible factor (HIF) via wnt-pathway signaling on oligodendrocyte precursor cells affects both white matter integrity (axonal loss) and angiogenesis. HIF works in the same way in the white matter as VEGF does in the retina, triggered to activate angiogenesis by hypoxic environment and downregulated in a hyperoxic environment¹⁹³.

The preterm rabbit pup model

The preterm rabbit pup model of IVH was first described in 1982. The rabbit pups have a germinal matrix and develop spontaneous IVH similar to that of preterm infants¹⁹⁴. The post-hemorrhagic process is similar as well regarding development of post-hemorrhagic hydrocephalus and abnormal neurological function similar to CP⁵⁴. Studies on the developing brain in other animal models such as mice and rats are performed in fullterm pups that have an immature brain and most of the brain development takes place during postnatal development. This obviates the effects of preterm birth *per se* and the early loss of placental connections.

The preterm rabbit pup model is one of few animal models that can be used to study the preterm brain in preterm born animals since rabbit pups have a relatively late cerebral development but an early lung development which enables survival from day 29 of gestation (term is day 32). On day 29 the cerebral maturation in the preterm rabbit pups corresponds to brain maturation of a human infant around GW 28. The cerebellar maturation on day 29 is more immature and corresponds to the cerebellum of a human infant at about GW 22 and at P9 to that of the full term infant (translatingtime.org)¹⁹⁵. Fig 3.

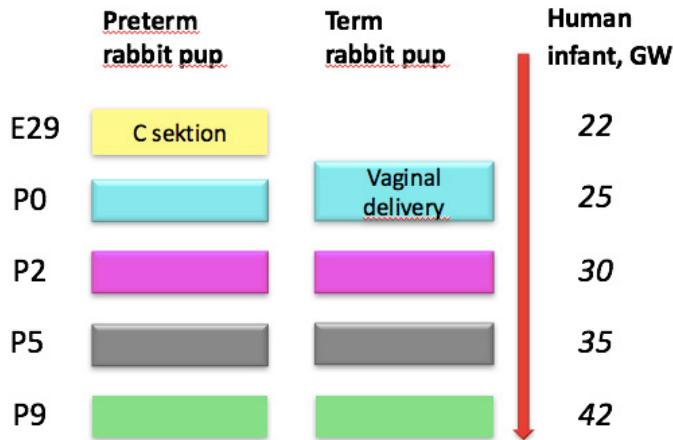


Figure 3. The development of the preterm rabbit pup cerebellum compared to that of the human.

The numbers on the left represent age of the preterm rabbit pup were E29 is the birthday, P0 is the birthday of term pups and corresponds to term corrected age for the preterm pups. The numbers on the right denote corresponding maturational age in the human fetus according to the translatingtime.org model.

Substantial improvements have been made with the rabbit pup model from the time of the preterm rabbit pup study in paper I to the study in paper IV. In the first study the preterm rabbit pups were cared for in an incubator and hand fed with a feeding tube twice a day. In the last study we have implemented the wet nurse model. A wet nurse is a rabbit that has given birth to her own pups the previous day and all but 2 of her own pups are removed on postnatal day 1 and replaced with the preterm pups. The preterm rabbit pups are then housed and fed with and by their wet nurse. The implementation of the wet nurse model has increased survival and improved weight development.

The application of high-frequency ultrasound has allowed for non-invasive evaluation of the preterm rabbit pup brain and of induced IVH. The size and development of IVH progress is monitored and evaluated with high spatial resolution using high-frequency ultrasound¹⁹⁶.

Preterm rabbit pups have only 2 small accessible veins and are extremely sensitive to stress and really need minimal handling. This makes repeated blood evaluation almost impossible which is why all of the blood evaluations are done at one time-point at termination.

Hypothesis and aims

The main question addressed in this thesis is why the cerebellum is smaller in preterm children compared to that in term born children and which neonatal events affect cerebellar development?

The general hypothesis is that preterm birth *per se* disturbs normal cerebellar development causing underdevelopment and that certain events following preterm birth, either complications or treatments, may aggravate the underdevelopment.

The overall aim is to evaluate how preterm birth in association with potentially damaging events can affect cerebellar development.

I. Hypothesis

Preterm birth and the subsequent loss of placental support with a resulting decrease in circulating levels of IGF-1, may be essential and partly causal, in the decreased cerebellar growth observed in very preterm infants.

Aim

To evaluate if preterm delivery in rabbit pups results in decreased circulating levels of IGF-1, and if preterm delivery *per se* would result in maturational changes in key elements of the developing cerebellum.

II. Hypothesis

Cell-free Hb and its degrading products are causal in cerebellar underdevelopment following IVH and therefore scavenging of cell-free Hb could reverse/restore the damage.

Aim

To evaluate the damaging effects of cell-free Hb on the developing cerebellum and if the effects are reversible by administration of a Hb scavenger.

III. Hypothesis

ROP is a continuous process of impaired vascular and neuronal retinal development, a neurovascular disease. Therefore the presence of any stage of ROP is associated with impaired brain growth and development.

Aim

To evaluate the relationship between the presence of any stage of ROP, brain growth and later developmental outcome at 2 years of corrected age in preterm infants.

IV. Hypothesis

Caffeine administered following preterm birth may modify the endogenous IGF-1 system by altering hepatic production of IGF-1, the primary source of circulating IGF-1. Profound changes in circulating IGF-1 may be reflected in altered regulation of the IGF-1R in key cerebellar cell populations.

Aim

To evaluate the effect of treatment with caffeine on hepatic and circulating IGF-1 and cerebellar expression of the IGF-1R in preterm rabbit pups. Secondly, to evaluate the effect of caffeine on key cell populations in cerebellar development.

Material and methods

Preterm animals

Paper I, II and IV

The preterm rabbit pup model

Paper I and IV

The animal protocol was approved by the Swedish Animal Ethics Committee in Lund. The experimental rabbits used were a half-breed between the New Zealand White and Lop.

The preterm rabbit pups were delivered by caesarean section at gestational day 29 (term 32 days) after the does were anesthetized with intravenous (i.v.) propofol (5 mg/kg) and given local anaesthetic in the abdominal wall using lidocaine with adrenaline (10 mg/mL + 5 µl/mL). After birth, the pups were dried and placed in a closed infant incubator with humidified air (60%). At 2 hours of age, the pups were weighed, marked and fed.

The term pups were delivered by spontaneous vaginal delivery at term (gestational day 32), and then nursed and fed by their lactating doe. All pups were weighed once daily.

The preterm rabbit pup model with IVH

Paper II

In paper II a preterm rabbit pup model of IVH was used. The rabbit pups were delivered and taken care of as described above for preterm rabbit pups. To induce IVH the preterm rabbit pups were injected intraperitoneally (i.p.) with 50% (v/v) sterile glycerol (6.5 g/kg; Teknova, Hollister, CA, USA) at 2 hours of age to induce intracerebral hypotension due to hyperosmolality and thus causing rupture of the small vessels in the germinal matrix.

Housing

After birth the preterm pups were placed and cared for in a closed infant incubator with humidified air. The conditions used in the different studies varied somewhat and were accordingly:

Paper I: initially at day 1: 37°C and 70% humidity; day 2, 36°C and 60% humidity, and thereafter at 35°C and 50% humidity.

Paper II: at 34-35°C and ambient humidity throughout the study.

Paper IV: The preterm pups were nursed by a wet nurse in her cage.

Feeding

Paper I: The preterm pups were hand-fed with kitten-milk replacement formula (KMR; PETAG Inc., USA) using a 3.5 Fr feeding tube, with the first feed at 2 hours of age at a volume of 1 mL and subsequently every 12 hours increasing each meal by 0.5 mL.

Paper II: The same feeding strategy as in paper I with the following changes: the first feed was at a volume of 2 mL and subsequently every 12 hours increasing each meal by 1.0 mL.

Paper IV: The preterm pups were hand fed the first meal with kitten milk as in the previous study. The wet nurse carried out all additional feedings.

Mortality/Survival

Paper I: Results from 64 rabbit pups from 16 litters were presented in the study. Mortality was 38% in the preterm group and 0 in the term group. Overall survival 62%.

Paper II: Results from 59 rabbit pups from 9 litters were presented in the study. Mortality was 40%. Overall survival 60%.

Paper IV: Results from 53 rabbit pups from 6 litters were presented in the study. Mortality was 38% in the control group but 14% in the studygroup. Overall survival was 75%.

Caffeine/vehicle administration

Paper IV

Study medication was administered daily with an oro-gastric tube with either caffeine or vehicle solution using a 3.5 French feeding tube until day of termination.

The caffeine used was Peyona® (20 mg/ml, Chiesi Farmaceutici) and administered at a concentration of 1mg/ml diluted in NaCl (9 mg/ml, Fresenius

Kabi). Day 1 the pups received a loading dose of 20 mg/kg and thereafter a daily maintenance dose of 5 mg/kg/d. Vehicle solution was NaCl (9 mg/ml) and administered in volumes corresponding to that of Peyona® in the treatment group.

Ultrasound imaging of the brain

Paper II

Ultrasound of the brain was performed at 6 hours of age to grade the severity of the IVH and detect SAH and daily thereafter using the high resolution VisualSonics Vevo 2100 (VisualSonics Inc., ON, Canada) with a MS 550D 40 MHz transducer. Animals with IVH at 6 hours were included in the IVH group, and those without detectable IVH at all time-points were included in the control group.

SAH was confirmed in all pups with IVH with visible presence of hemorrhagic CSF covering the cerebellar cortex at removal of the brain from the skull. None of the control pups exhibited macroscopic signs of SAH. None of the cerebellar samples in pups with or without IVH exhibited signs of primary cerebellar hemorrhage.

Intraventricular injections

Paper II

Pups with IVH were randomized into IVH, IVH+Hp, or IVH+Vehicle groups. Pups in the IVH+Hp and IVH+Vehicle groups received an ultrasound-guided intraventricular injection at 8 hours of age of either 20 µl of human Hp (50 mg/ml, Bio Products Laboratory, London, UK) or 20 µl of vehicle solution (9 mg/ml NaCl, Fresenius Kabi, Lake Zurich, IL, USA), using 27 G Hamilton syringes (Hamilton Robotics, Reno, NV, USA).

Blood sampling for serum IGF-1 concentrations

Paper I and IV

Blood was sampled from all pups through cardiac puncture after sedation with isoflurane inhalation prior to *in vivo* perfusion-fixation of the brain. Blood was collected in serum tubes and centrifuged at 1,000 xg for 10 minutes at room temperature. The serum was then transferred into new tubes and stored at -80°C until analysis. The rabbit pup serum concentration of IGF-1 was determined using the human IGF-1 ELISA from Mediagnost (Reutlingen, Germany). The manufacturer has proved this assay applicable for rabbit serum samples.

Brain tissue sampling and processing

Paper I, II and IV

Perfusion-fixation of the brain was performed by cardiac cannulation following thoracotomy and infusion of 0.9% saline followed by 4% paraformaldehyde (PFA

buffered with phosphate buffer saline (PBS) 0.1 M, pH 7.4). After complete perfusion, the cerebrum and cerebellum were carefully extracted from the skulls and immersed in 4% PFA. A change to fresh PFA was done after 3–6 hours.

The brains were fixated in 4% PFA for 48 hours. Thereafter, they were dehydrated, cleared, and infiltrated with paraffin automatically in a TISSUE-TEK V.I.P. (Miles Scientific Corp., Newark, NJ, USA) and embedded in paraffin blocks. The cerebellum was sectioned, and 4µm sections in the parasagittal plane at the level of the dentate nucleus were made (Leica, RM2255 Microtome) and mounted on microscope slides and dried at 37°C for 12–16 hours.

Immunohistochemical staining for cerebellar development

Paper I and II

Following deparaffinization, the sections were antigen-retrieval pre-treated by boiling in 0.05 M boric acid buffer (pH 8.0) followed by incubation with antibodies towards the antigens described in table 1 according to material and methods in paper I and II.

Table 1.
Primary and secondary antibodies used.

Epitope	Host species	Code	Dilution	Manufacturer
Calbindin	Mouse	CB-855	1:200	DBS
Calbindin	Mouse	Ab82812	1:25	Abcam
Ki67	Mouse	M7240	1:100	Dako
Cleaved Caspase-3	Rabbit	#9661	1:100	Cell signaling
GFAP	Rabbit	Ab16997	1:150	Abcam
Olig 2	Mouse	MABN50	1:1000	Millipore
Iba 1	Rabbit	CP290	1:200	Biocare
Shh	Rabbit	Ab73958	1:200	Abcam
Hb	Goat	GWB-F26D80	1:500	GenWay Biotech
Hp	Chicken	GWB-431F62	1:1000	GenWay Biotech
IGFR1	Goat	AF-305-NA	1:25	R&D systems

Secondary antibodies	code	application	Dilution	Manufacturer
Donkey anti Goat IgG (Alexa Fluor 488)	705-546-147	IGFR1	1:200	JIR
Donkey anti Mouse (Rhodamine-Red)	715-296-150	Calbindin, Ki67	1:200	JIR

Time-points for analyses:

In paper I: E29, P0, P2, P5, P9 and evaluation for cerebellar white matter damage at P2 and P5.

In paper II: P0, P2 and P5.

In paper IV: P3, P4, P5 and P6

Histological Analysis of the EGL

Paper I and II

In paper I and II analysis of the EGL was performed in 4 predefined regions: the inner and outer portion of lobule V, and the inner and outer portion of lobule IX, respectively. These regions were chosen as the regions with possible maturational differences in EGL proliferation and in subsequent width. Measurement of the width of the proliferative EGL, as constituted by Ki67-positive cells, was performed by using the x40 objective lens on the Leica DMRX microscope. The average of the 4 respective measured widths was calculated for each pup. Ki67-neg cells were regarded as differentiated, and were counted over an area of 100µm. Using the Leica Q500 image analysis system of the microscope, the area of calbindin-positive-stained cells was determined in relation to the area of the molecular layer. Thus, calbindin staining was expressed as percentage positive area in relation to a standardized area of the molecular layer. Nonspecific background staining was taken into consideration.

In paper I qualitative evaluation was performed of Purkinje cell and Bergmann glia morphology at repeated postnatal ages in both the preterm and term groups. Distribution and the presence of immunoreactivity for cleaved caspase-3 and Shh were described qualitatively. Cerebellar white matter impairment was evaluated in preterm and term pups by HE staining. We further performed qualitative and quantitative analysis of immunostaining for Ki67, Olig2, and Iba1, for determination of cell proliferation and presence of oligodendroglial and microglial cells in preterm and term pups.

Immunofluorescent labeling for Hb and Hp

Paper II

Double immunofluorescence labeling of Hb together with human Hp was performed to simultaneously investigate the presence and distribution of both encapsulated erythrocytes and cell-free Hb within the cerebellum and to elucidate whether the intraventricularly injected human Hp could reach the cerebellar brain regions

containing Hb (preferentially the cell-free Hb) in the IVH rabbit pups as described in materials and methods of paper II.

Immunofluorescence for IGF-1 and IGF-1R

Paper IV

Double immunofluorescence was performed for epitope detection of IGF-1R in combination with Calbindin or Ki67 as described in material and methods of paper IV, Table 1. Analysis was performed in lobule IX and X.

RNA Isolation and Real-Time PCR

Paper II and IV

In paper II and IV total RNA was extracted from the cerebellar tissue (paper II) and liver (paper IV) of the rabbit pups using the NucleoSpin RNA/protein extraction kit (paper II) or RNeasy Mini Kit supplied by QIAGEN (Germantown, MD, USA) as described in material and methods of paper II and IV.

Reverse transcription was performed according to the manufacturer's instructions on 1µg total RNA using iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA) and the RT² qPCR Primer Assay (primer from QIAGEN, Germantown, MD, USA) was used to quantify mRNA expression of heme oxygenase 1 (HO-1), and expression was analysed using iTaq Universal SYBR Green Supermix (Bio-Rad). Amplification was performed as described by the manufacturer (Bio-Rad) for 40 cycles in an iCycler Thermal Cycler (Bio-Rad), and data were analysed using iCycler iQ Optical System Software (Bio-Rad). Data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH, primer from QIAGEN), with fold change values calculated by normalizing against control animals.

Quantitative analysis

Paper IV

Quantitative analysis were performed using ImageJ 1.51u. A macro code was developed to perform the analysis on each image obtained for the project evaluating Ki67 (red channel), Calbindin (red), IGF-1R (green) and DAPI (blue).

A fixed threshold was used for the red and green channel to allow for comparison of each individual image. A variable threshold was used for the blue channel to get the best identification of cell nuclei. The total number of cell nuclei are kept and

used to calculate the area per cell for each image. For the Ki67 marker a logical and operation with the DAPI channel identifies the number of cells that are positive for Ki67.

Preterm infants

Paper III

Study population

The original prospective study included 64 very preterm infants born at the neonatal intensive care unit (Lund, Sweden) between January 2005 and May 2007, where IGF-1 concentrations from birth until term age in relation to growth, MRI-estimated brain volume, and developmental outcome were evaluated^{51,56,64}. Inclusion criteria were GA < 31 weeks at birth, absence of major congenital anomalies, and written informed parental consent. All pregnancies were dated by ultrasound at 17–18 GW.

52 infants completed the study until term age, 51/52 underwent MRI at term age and 49/52 received follow-up examination at 2 years corrected age. Of the 64 recruited infants, 9 did not survive until term age and the parents of 3 infants chose to leave the study.

Out of 52 infants, 33 had no ROP, 9 had mild ROP (stage 1-2), and 10 had severe ROP (stage 3 or more, none of the infants had ROP > stage 3). Of these 10 infants with severe ROP, 9 received laser treatment.

The study was approved by the regional ethical review board of Lund, Sweden and adhered to the tenets of the Declaration of Helsinki.

Clinical data

Weight standard deviation score (SDS) at birth was calculated from a Scandinavian intrauterine growth curve based on fetal weights estimated by ultrasound¹⁹⁷. Cumulative dose of administered (mg/kg) hydrocortisone and betamethasone were registered until PMA of 35 weeks. Total steroid exposure was estimated by converting the betamethasone dosage into hydrocortisone equivalents (1:40). Bronchopulmonary dysplasia was defined as a requirement for supplemental oxygen at PMA of 36 weeks. Septicemia was defined as the presence of positive blood culture and concomitant increased levels of C-reactive protein.

Nutritional regime and calculation of intake

Maternal and donor breast milk was analyzed weekly for protein and energy content and enteral and parenteral daily intakes of protein (g/kg/d) and energy (kcal/kg/d) were prospectively calculated from birth until at least PMA of 35 weeks.

Cerebral ultrasound

Cerebral ultrasound was performed on days 1, 3, and 7; at 3 and 6 weeks of age; and at term. Severe intracranial hemorrhage was defined as the presence of IVH grade III or parenchymal hemorrhage. White matter damage was defined as the presence of periventricular echodensities or cysts that persisted for >7 days. Severe brain damage was defined as severe intracranial hemorrhage and/or white matter damage.

ROP examination

ROP screening began at 5–6 weeks of age, but not before PMA of 31 weeks. The infants underwent retinal examinations through dilated pupils biweekly to once weekly depending on ROP severity, either until the retina was fully vascularized or the condition was considered stable. ROP was classified according to the International Classification of Retinopathy of Prematurity¹²⁶, and treatment followed the recommendations of the Early Treatment for Retinopathy of Prematurity Cooperative Group¹⁹⁸.

MRI

MRI was performed on a 3-Tesla Siemens Magnetom Allegra head scanner (Siemens AG Medical Solutions, Erlangen, Germany) in 51/52 infants at term age, mean (SD), 40.1 (0.6) gestational weeks. The protocol consisted of a three-dimensional T1-weighted (T1w) magnetization-prepared rapid gradient echo (MPRAGE) a T2-weighted (T2w) turbo spin echo sequence and a proton density weighted (PDw) turbo spin echo sequence. A three-channel (T1w, T2w, PDw) dataset was created. A template modified, statistical classification algorithm (nearest neighbor) was used for image classification into myelinated white matter (MWM) and unmyelinated white matter (UWMV), total gray matter (GMV), and CSF, based on signal intensities on these three channels. The voxels of every tissue class were summed to calculate the tissue volumes. Total brain volume (TBV) was defined as the total volume of GMV, MWM, and UWMV, including the cerebellum. The cerebellar volume, including the cerebellar peduncles, was measured by manual outlining on both T1w and T2w images, using an image analysis tool (www.slicer.org). All segmentations were performed by the same person. We calculated tissue volumes of TBV, GMV, UWMV in 46 infants and cerebellar volume in 51 infants

Assessment at 2 years corrected age

A psychologist assessed developmental outcome in 49/52 infants at the mean (SD) corrected age of 24.6 (0.8) months by means of the Bayley Scales of Infant Development (BSID-II), with two different index scales, the MDI and the PDI⁵⁶.

Statistics

All statistic analyses were performed with IBM SPSS for Microsoft Windows (IBM, Armonk, NY, USA), different versions with time. $P < 0.05$ was considered significant.

Paper I

Differences between groups and time-points were analyzed with the Mann-Whitney U test. Correlations between continuous variables were analyzed with regression analysis and adjustment for other variables was performed with multivariate regression analysis.

Paper II

Results are presented as medians (ranges) and displayed as boxplots. Comparisons between unrelated groups were performed with the Mann-Whitney U test. Comparisons between multiple groups were made using the Kruskal-Wallis test followed by pairwise comparison with significance values adjusted for multiple comparisons.

Paper III

Univariate analyses of differences between groups were assessed with the Mann-Whitney U test or Chi-square test as appropriate. Correlations between continuous variables were evaluated with the Spearman rank correlation coefficient.

Adjustment for other variables was performed with multiple linear regression analysis where GA, BW and gender were included as independent variables in all models. In addition, all variables exhibiting significant univariate associations with the respective outcome variables were included.

Paper IV

Values of serum IGF-1 and hepatic mRNA for IGF-1 are presented as box plots displaying median, and 25th and 75th percentiles. One-way ANOVA was used to evaluate the effect of treatment on serum IGF-1, mRNA for IGF-1 and for weight at multiple time-points. Multiple linear regression analyses with serum IGF-1 and

mRNA IGF-1 respectively as dependent variables was used to evaluate the effect of treatment adjusting for weight and postnatal age.

Ethical considerations

All the studies were approved by the Regional Research Ethical Committé in Lund.

Clinical studies

In the clinical study which provided the study material for paper III, parental informed consent was the rule for inclusion in the study. Information was given to parents prior to delivery to allow for sufficient time for parental consideration concerning study participation. Clinical studies in preterm infants are a challenge. The preterm infant can not give consent to participation and therefore the informed consent relies on the parents. It is considered an advantage to be able to give study information to parents prenatally since the postnatal period is a period of parental emotional and psychological stress which can compromise the retrieval of information.

Animal studies

Animal studies are still essential in order to further knowledge in basic biology as well as to retrieve knowledge with an immediate potential for translation to clinical medicine such as novel therapies and related pharmaco-kinetics and toxicity issues.

The rights of animals must be respected. The animal model is chosen with the aim of optimizing representation of the clinical context. A study must have a clear purpose and aim and requires careful planning for the number and types of procedures and to minimize the number of animals needed for a study. The animals should be treated with the same precautions and respect as human patients are, with minimal handling and always provide the appropriate sedation and analgesia as needed.

With careful planning a research team can frequently perform an animal study with more than one purpose. This was particularly the case in paper IV. Careful planning permitted the study of multiple outcomes beyond those described in the present thesis.

Results and comments

The effect of preterm birth, IVH and caffeine on cerebellar development in preterm rabbit pups.

Survival

From the first study in preterm rabbit pups and to the last study there is a relative increase in survival. The mortality in preterm rabbit pups has been reported as 20% in rabbit pups after a glycerol injection and 30% in those who develop IVH in Ballabhs research lab ⁵⁴. This mortality rate has been questioned by Traut *et al* who had double the mortality rates reported by Ballabh ¹⁹⁹. Both research groups use other rabbit breeds than we do. With time, improvements have been made in the preterm rabbit pup model to establish better nutritional status and to increase survival and improved handling of the animals by a dedicated animal technician. The mortality in paper I was 38% in the preterm pups. There were indications of undernutrition which resulted in increase of the daily feedings and housing adjustments of temperature and humidity in paper II. The mortality in that study was the same, 38% but those pups had IVH which is known to increase the mortality rate as mentioned previously. This could therefore be interpreted as improved survival after adjustments. In the most recent study, the caffeine study, the wet nurse model was implemented and the overall mortality was reduced to 25%. The mortality in the control group was still 38% but significantly reduced in the caffeine group to 14% ($p=0.022$). This suggests that treatment with caffeine increases survival in the preterm rabbit pups. It is conceivable that an apnea-preventing effect may have contributed to the observed decreased rate of mortality.

Postnatal growth

Postnatal growth was evaluated in the preterm rabbit pups and compared to that of term born pups in paper I. Preterm birth in rabbit pups was associated with profound growth restriction and the mean weight was significantly lower in the

preterm pups compared to the term pups at all time-points during the study period (all $p < 0.05$). Fig 4A. Relative increase in weight was 3-fold higher in term pups compared to preterm pups. No catch-up in weight was noted in preterm pups during the study period which could be caused by a suboptimal nutritional strategy.

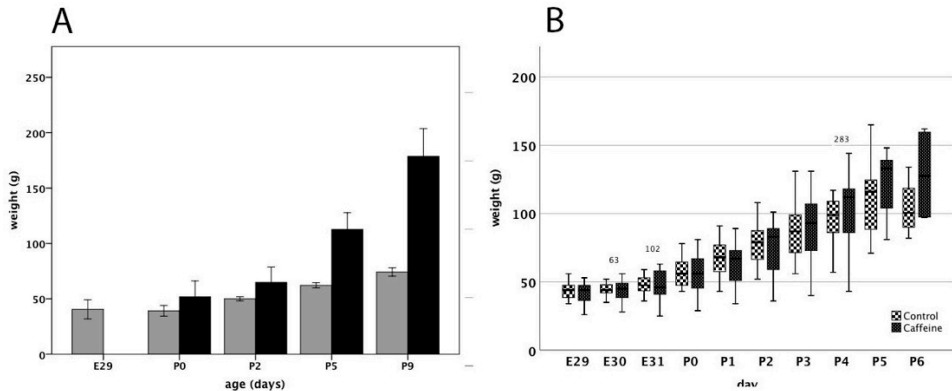


Fig 4. Postnatal weight development in preterm and caffeine-exposed rabbit pups.

Fig A shows mean weight in relation to postnatal age in preterm rabbit pups (gray) compared to term rabbit pups (black), with a significant difference at all time-points ($p < 0.05$). Bars represent group means and error bars 1 SD. Fig B shows weight development in preterm rabbit pups treated with either caffeine (black) or vehicle solution (squared black and white). There was no significant difference between the groups at any time-point. Box plots include median values and 25th and 75th percentiles.

In paper IV, the growth of preterm rabbit pups that had been treated with caffeine was compared to control preterm pups that received vehicle solution. Caffeine did not have any discernible effect on growth, Fig 4B. Thus, no growth retardation was noted in the caffeine-exposed pups similar to that described by others in caffeine-treated preterm human infants¹⁵³.

An interesting observation was made when the growth pattern of the preterm rabbit pups in paper I was compared to that of the preterm rabbit pups in paper IV. With the introduction of the wet nurse model in paper IV, the preterm rabbit pups had a similar nutritional strategy as the term control pups in paper I and exhibit a very similar increase in postnatal weight. In summary, modification of nutritional strategy obliterated the influence of differences in GA at birth on postnatal weight development

The Insulin-like growth factor system

Circulating IGF-1 and hepatic mRNA expression for IGF-1.

The levels of circulating IGF-1 were evaluated in the preterm rabbit pups compared to term rabbit pups in paper I and in the caffeine-treated preterm pups compared to the vehicle-treated preterm pups in paper IV.

Paper I. Mean circulating levels of IGF-1 were significantly lower in the preterm rabbit pups at all time-points compared to the term rabbit pups (all $p < 0.05$), Fig 5A. The levels increased slowly in the preterm group but at P9 there was a fall in serum IGF-1 levels in the preterm group, most likely associated with nutritional status, Fig 5A.

Paper IV. Circulating IGF-1 and hepatic expression of mRNA for IGF-1 were not affected by caffeine treatment ($p = 0.90$ and $p = 0.29$ respectively) at any given time-point in the study. Fig 5B-C.

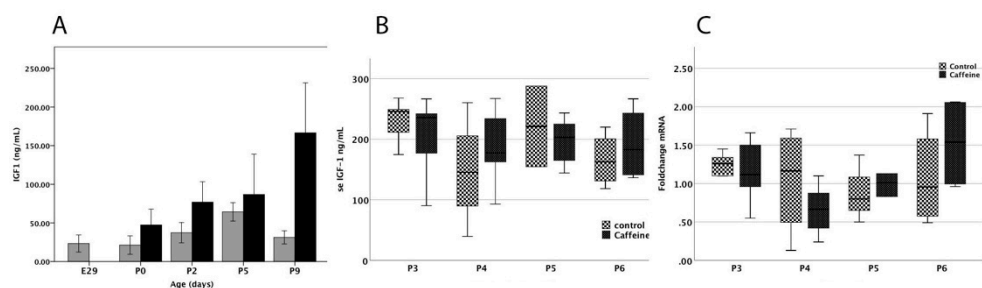


Fig 5. The IGF-1 system in relation to preterm birth and exposure to caffeine in rabbit pups.

Fig A shows serum IGF-1 levels in the preterm rabbit pups (gray) and term pups (black) in relation to postnatal age. Bars represent group means and error bars correspond to 1 SD. Fig B shows serum IGF-1 levels in relation to postnatal age in caffeine-treated preterm rabbit pups (black) compared to vehicle-exposed pups (squared black and white). Fig C shows hepatic expression of mRNA for IGF-1 in relation to postnatal age in the same experimental groups as in panel B. Boxplots denote medians and 25th and 75th percentiles.

Concentrations of serum IGF-1 were highly correlated with weight ($r^2 = 0.89$, $p < 0.001$) and were not affected by caffeine exposure ($p = 0.876$). A similar relationship was observed between weight and hepatic mRNA for IGF-1 with no effect of caffeine exposure ($p = 0.974$). Circulating serum IGF-1 values correlated significantly to hepatic expression of mRNA for IGF-1 ($r^2 = 0.38$, $p < 0.001$).

In paper IV early postnatal time-points were not evaluated. Levels of serum IGF-1 were considerably higher than those observed in preterm pups in paper I. Thus, the modified nutritional strategy in paper IV in comparison to that in paper I had a

strong impact on serum IGF-1, very similar to that previously discussed on weight development.

Cerebellar distribution of IGF-1R

Besides circulating IGF-1 and hepatic expression of mRNA for IGF-1 the cerebellar expression of the IGF-1R was evaluated in paper IV. Staining for IGF-1R was only performed at one time-point, P4, which limits the interpretation of the results to the location of the expression in comparison to what is known from other experimental studies and to comparison between the caffeine-exposed pups and the control pups.

Quantitative analysis showed no difference in IGF-1R immunoreactivity between caffeine-exposed pups and control pups ($p = 0.49$).

IGF-1R was clearly present in the proliferative part of the EGL surrounding proliferating Ki67 positive granule precursor cells, Fig 6 A-D. Positive staining for the IGF-1R was also detected in the molecular layer among the axons of the granule neurons and the dendrites of the Purkinje cells and the Bergman glia and to a lesser extent in the IGL. Positive staining for IGF-1R was present in cell membranes or in the adjacent extracellular space and never detected intracellularly, neither in the granule cells nor in Purkinje cells. Bondy *et al* made a similar observation at the same stage of development and in the absence of IGF-1R noted a high expression of the ligand, IGF-1. At later developmental age the same authors observed a high co-localization of the receptor and the ligand in Purkinje cells¹⁷⁹.

Since IGF-1 as a ligand exerts its function via the IGF-1R, these results are highly relevant for addressing the role of IGF-1 in the proliferation in the EGL. At this time-point serum IGF-1 is still increasing and proliferative cells more active than differentiated cells that should be migrating out of the EGL inward to form the IGL, guided on their way by the Bergman glia. The findings of expression of IGF-1R in the cerebellum at P4 are consistent with previous description of the IGF-1R in postnatal rat brain¹⁷⁹. Studies of the ligand IGF-1 at similar postnatal age have shown a preferential expression in differentiated cells²⁰⁰, but the expression of the IGF-1 ligand is not included in our study.

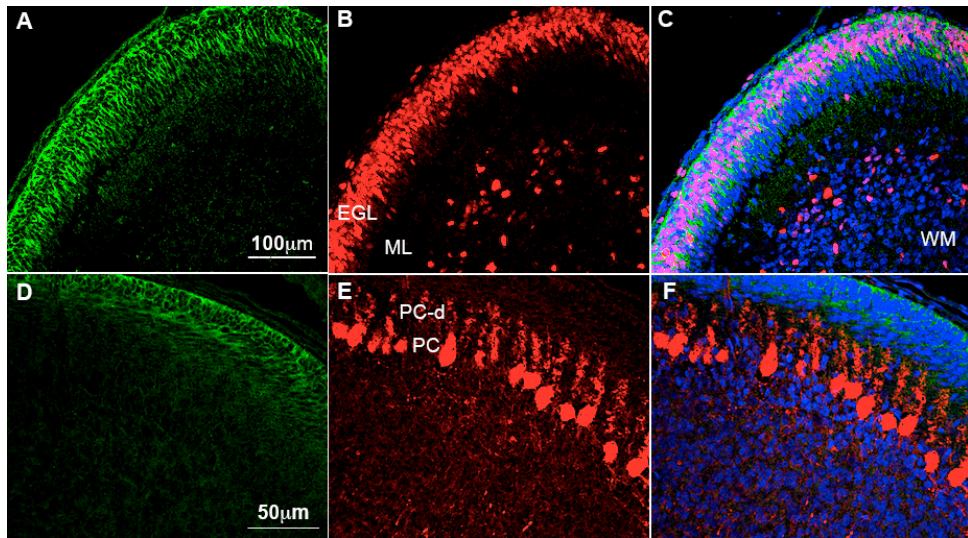


Fig 6. Immunofluorescent staining for key elements in cerebellar development.

A and D. IGF-1R (green) exhibits the strongest expression in the outer part of the external granular layer (EGL) but is also expressed in the inner part of the EGL and in the molecular layer (ML) and the forming IGL. Staining for IGF-1R is observed in cell membranes and/or in the adjacent extracellular space around EGL granule precursor cells. Additionally positive IGF-1R staining was observed in the ML with a radial distribution. There is no visible intracellular expression. **B** shows staining for Ki67 corresponding to proliferating cells (red). The cell bodies of the granule precursor cells in the outer EGL layer stain positively. Several cells stain positively in cerebellar white matter (WM). **C** Merged panel of IGF-1R (green), Ki67 (red) and DAPI for cell nuclei (blue). Staining for IGF-1R (green) co-localizes with proliferating Ki67 positive cells (pink) in the outer EGL. **E** Calbindin staining (red) showing a monolayer of Purkinje cell bodies (PC) and axons with dendritic arborisation (PC-d). **F** Merged panel of IGF-1R (green), calbindin (red) and DAPI for cell nuclei (blue). Absence of co-localisation between IGF-1R and calbindin-positive Purkinje cells.

External granular layer development

Key elements in EGL development were analyzed in paper I, II and IV. The general outline of the EGL development is shown in Fig 6 and previously discussed.

Proliferation and differentiation following preterm birth

Paper I. The proliferative (Ki67 positive) portion of the EGL was decreased in the preterm group at P2 compared to term pups ($p = 0.01$), Fig 7A. P2 was the only time-point that exhibited a significant difference. When analysing the proliferative pattern over time in the preterm and the term pups the pattern is similar, proliferation starts to decline 5 days after birth in both groups and since the preterm pups are born 3 days earlier than the term pups this decline occurs on P2 as compared to P5 in the term pups.

The pattern over time of differentiated (Ki67 negative cells) is the same in both groups with no significant difference at any time-point which could be interpreted

that timing of differentiation is less modified by preterm birth than that of proliferation, Fig 7B.

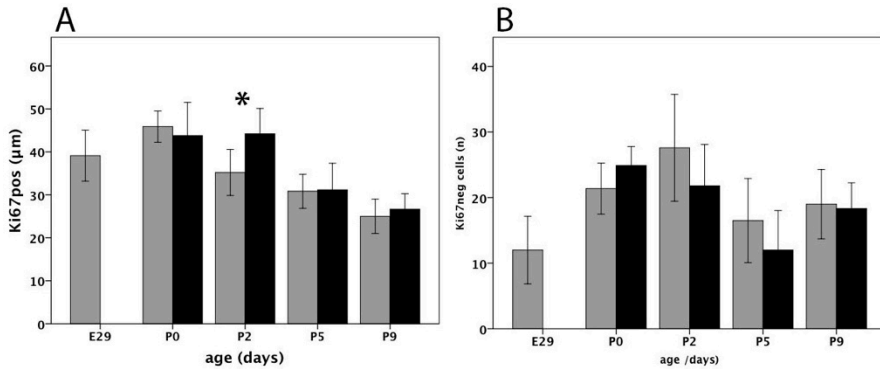


Fig 7. Proliferating and non-proliferating cells in the EGL in relation to postnatal age.

Fig A. Mean values (width) of proliferating, Ki67 positive cells in relation to postnatal age. Fig B. Mean number of differentiated, Ki67 negative cells in relation to postnatal age. Preterm pups = gray columns, term pups = black columns. Bars represent group means and error bars correspond to 1 SD.

Paper II. Following IVH there was a significant decrease in the width of the proliferative (Ki67 positive) portion of the EGL at the latest time-point evaluated P5 ($p = 0.017$) compared to pups with no IVH. No significant difference was seen at the earlier time-points P0 and P2.

Paper IV. Administration of caffeine to the preterm rabbit pups had no significant quantitative effect on Ki67 positive proliferating cells of the EGL.

Purkinje cell maturation

Paper I. Purkinje cells exhibited decreased calbindin staining in the preterm rabbit pups compared to term pups at P0 ($p = 0.003$), P2 ($p = 0.004$), and P5 ($p = 0.04$). Purkinje cell morphology was clearly affected with reduced arborization and dendritic spines in the preterm group at P0 and P2, compared to term pups. At later time-points the Purkinje cell development in the preterm pups caught up with that of the term pups with no significant differences observed.

Paper II. Similar results as in paper I were observed after IVH with delayed Purkinje cell maturation with smaller neuronal cell bodies and underdeveloped dendrites at P0 ($p = 0.015$) and P2 ($p = 0.026$) in preterm pups with IVH as compared to control preterm pups. Fig 8.

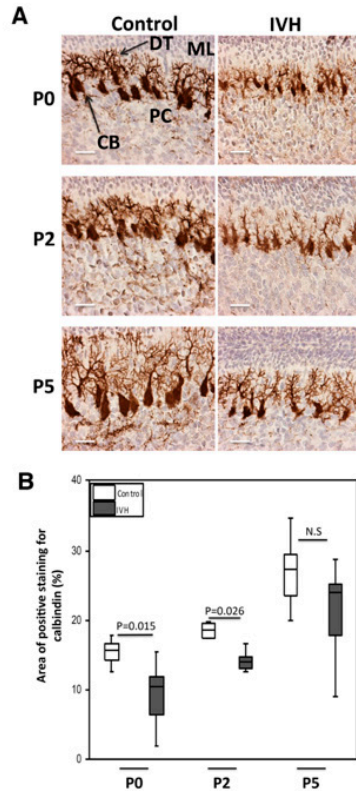


Fig 8. Impaired Purkinje cell maturation following IVH in preterm rabbit pups.

Fig **A**. Calbindin staining as a marker of Purkinje cell (PC) morphology. The IVH pups have reduced calbindin immunoreactivity and smaller cell bodies (CB), underdeveloped dendrites (DT) and reduced arborisation compared to control preterm pups at all time-points. ML molecular layer. Fig **B**. Quantification of calbindin staining at different postnatal time-points with significant reduction in the IVH preterm pups (gray boxplots) as compared to control preterm pups (white boxplots) at P0 and P2. Boxplots denote medians and 25th and 75th percentiles.

Paper IV. Purkinje cell morphology following caffeine administration was only evaluated at P4 for quantitative comparison between caffeine treated preterm pups and control preterm pups. There was no quantitative difference between the groups. Fig 6E shows the morphology of the Purkinje cells at P4.

Cerebellar white matter

Cerebellar white matter was not the primary focus in the experimental studies included in this thesis, but two important findings deserve mentioning.

In paper I, we observed cerebellar white matter damage in a subgroup of the preterm pups which was not associated with cerebral or cerebellar hemorrhage and

was not detected in any of the term pups. Further analysis of the affected areas revealed reduction in pre-oligodendrocytes ($p = 0.009$), decreased Ki67 positive proliferation ($p = 0.003$) and increased activation of microglia ($p = 0.04$). These findings are similar to those observed in periventricular cerebral white matter following IVH^{54,201}. The reason for this finding is unclear but could be a consequence of malnutrition, hypoxia or inflammation which are well described risk factors for white matter damage in the cerebrum²⁰².

In paper II, the rabbit pups with IVH had a significantly higher area of activated microglia in the cerebellar white matter at P0 ($p = 0.009$) and P2 ($p = 0.004$). This suggests that the deposition of cell-free Hb in the cerebellum may induce a microglial pro-inflammatory response with adverse effects on the immature oligodendrocytes and a subsequent cerebellar white matter impairment comparable to that described in cerebral white matter following IVH^{54,104,201}.

The observations in cerebellar white matter in paper I and II suggest that white matter development in the cerebellum is an important area for future studies on cerebellar development following preterm birth.

IVH, cell-free hemoglobin and the cerebellum

Cell-free Hb distribution following IVH

Cell-free Hb distribution was evaluated in the cerebellum at P0 in preterm rabbit pups 3 days after an IVH. Immunofluorescent staining revealed extensive deposition of RBCs in the subarachnoidal space surrounding the cerebellar lobuli following IVH which was not observed in control pups, Fig 9B+F. Labeled Hb was wide spread within the cerebellum. There was surprisingly little deposition in the EGL and more extensive deposition in the deeper cerebellar layers, the molecular layer and in the white matter.

An RT-PCR analysis of mRNA expression in cerebellar tissue of the major heme-degrading protein heme-oxygenase 1 (HO-1) was ten-fold higher in IVH pups compared to controls at P0 which further supported the existence of massive amount of cell-free Hb in the cerebellum following IVH.

Haptoglobin distribution

The haptoglobin (Hp) system is the scavenging system for cell-free Hb. Cell-free Hb binds to Hp forming an inert Hb-Hp complex which channels the Hb molecule for internalization and degradation by the CD-163 positive macrophages^{111,112}.

The cell-free Hb scavenger Hp was injected intraventricularly in the rabbit pups with IVH at 8 hours of age, about 2 hours after the diagnoses of IVH. The

distribution of Hp was analysed at P0, 3 days post IVH. No endogenous Hp was detected in any of the control pups, and was thus only detected in IVH pups that received the intraventricular injection. The Hp immunolabeling was widely distributed throughout large parts of the cerebellum, Fig 9K.

Double immunofluorescence labeling of Hp and Hb in these pups displayed a high degree of co-existence of human Hp and Hb in most regions, including the molecular layer and white matter, Fig 9L.

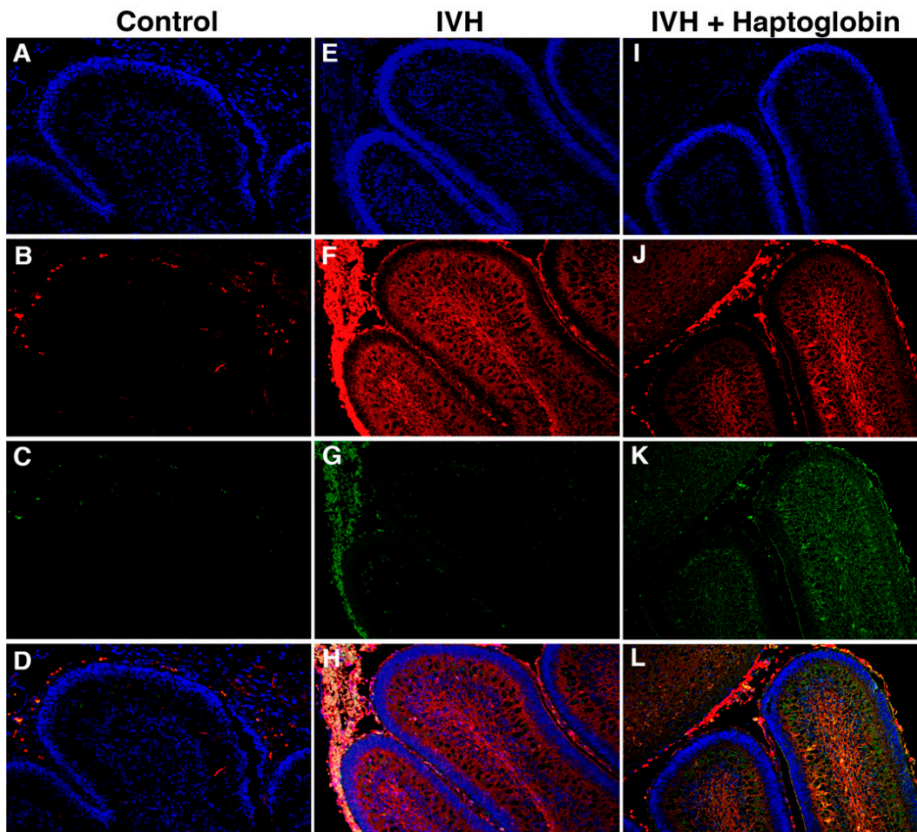


Fig 9. Immunofluorescent labeling of Hb and Hp following IVH in preterm rabbit pups.

Double immunofluorescence labeling of hemoglobin (Hb) (red) and haptoglobin (Hp) (green) together with a DAPI nuclear staining (blue), in animals with no IVH (Control), in animals with IVH (IVH), and in animals with IVH that received human Hp injections (IVH + Hp). **A-D** Control animal: Images **B**, **C** show the lack of Hb and Hp labeling. **E-H** IVH: In pups with IVH, the Hb labeling (red) was extensive, widely distributed in the molecular layer and white matter and to some degree in the EGL. Whole erythrocytes in the subarachnoid space surrounding the cerebellar lobuli were also intensely labeled and gave rise to green autofluorescence (**G**), observed as yellowish in the merged image (**H**). Hb labeling intermingled with dense nuclear regions (intense DAPI staining) appears as pink (bottom images). **I-L** IVH + Hp: **J** and **K** show immunofluorescence labeling of Hb (red) and human Hp (green) following intraventricular injection of Hp at E29. **J** shows the widespread distribution of cell-free Hb (red), corresponding to that in IVH animals (**F**), and the dominating co-existence of Hp in **K** (green), primarily in the molecular layer, white matter, and the EGL as shown in the merged image (**L**). Hp labeling was scarce in the subarachnoid space (**K**, **L**), in which Hb labeling of RBCs was extensive (**J**, **L**). Thus, the cell-free Hb and Hp are clearly distinguishable from the cell body-associated Hb labeling and autofluorescence. Scale bar = 50 μ m.

Intraventricular injection of Hp resulted in partial reversal of the damaging effects observed on Purkinje cell maturation following IVH, Fig 10 A-D. To strengthen the qualitative observation of the damage reversal a quantification analysis was done which confirmed the qualitative observation. There was no significant difference between the control pups and the IVH Hp group but a significant difference between the control pups and IVH vehicle group ($p = 0.024$), Fig 10 E.

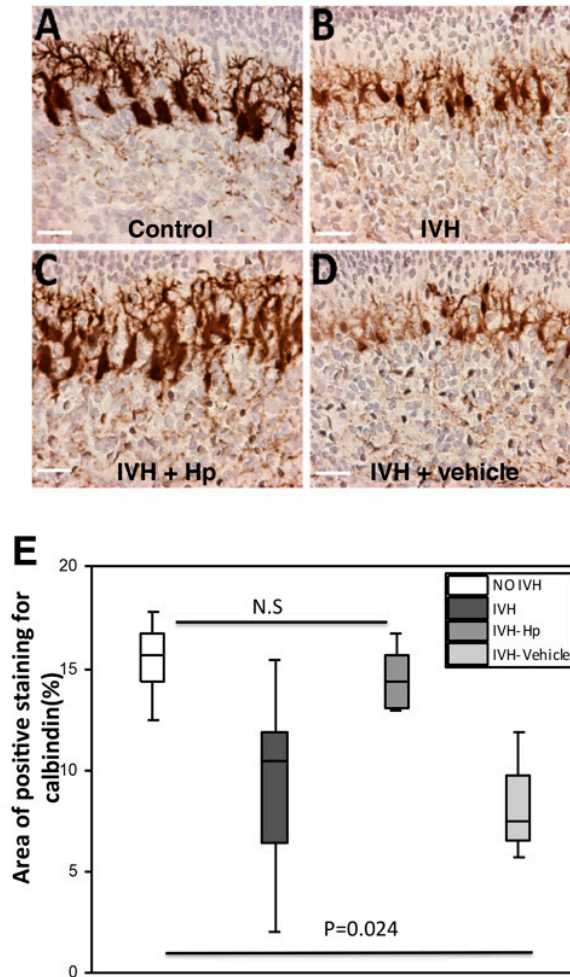


Fig 10. The protective effect of cell-free Hb scavenging on Purkinje cell maturation after IVH.

A–D. Following intraventricular Hp administration at P0, a higher intensity of calbindin immunoreactivity, relatively larger Purkinje cell bodies, and developed dendrites were observed in the Hp-administered IVH pups as compared to pups with IVH only or vehicle-treated IVH pups. Scale bar = 50 μ m. **E.** Quantification of Purkinje cell development at P0 of control pups (white bars, $n = 6$), IVH pups (dark gray bars, $n = 6$), and following intraventricular injection of Hp in pups with IVH (IVH + Hp, gray bars, $n = 6$) or vehicle solution (IVH + Vehicle, light gray bars, $n = 4$).

MRI studies in preterm human infants with IVH have shown infratentorial hemosiderin deposits on the cerebellar surface in the posterior fossa in up to 80% of preterm infants with IVH and disrupted cerebellar development without any cerebellar hemorrhage⁵⁰. Our study shows that there is an extensive deposition of cell-free Hb in the cerebellum following IVH with subsequent damaging effect on the proliferation and Purkinje cell maturation and that this damaging effect can be partially reversed with scavenging of cell-free Hb.

Retinopathy of prematurity, brain growth and developmental outcome in very preterm infants

Clinical risk factors for ROP, cerebellar growth and developmental outcome

Clinical risk factors for any ROP

The clinical risk factors for infants with any ROP were lower GA ($p < 0.001$), lower BW ($p < 0.001$), higher frequency of septicemia ($p = 0.002$) and a higher total steroid intake ($p < 0.001$) compared to infants without ROP, table 2.

Table 2.

Clinical characteristics with significant association to the presence of any retinopathy of prematurity ($n = 52$).

	No ROP ($n = 33$)	Any ROP ($n = 19$)	P value
GA, weeks, median (range)	27.4 (24.3-30.6)	25.0 (23.0-27.1)	<0.001
Birth weight, g, median (range)	970 (592-1716)	634 (348-854)	<0.001
Septicemia, n (%)	7 (21)	12 (63)	0.002
Total steroid intake, mg/kg, median (range) ^a	0 (0-112)	34 (0-105)	<0.001

^aCalculated hydrocortisone equivalents (mg/kg) from birth until postmenstrual age of 35 weeks.

Clinical risk factors for brain volumes

The risk factors for lower cerebellar and unmyelinated white matter volumes (UWMV) were GA (both $p < 0.001$), BW (both $p < 0.001$), septicemia (both $p = 0.02$) and a higher total steroid intake ($p = 0.001$ and $p < 0.001$). These risk factors correspond to those observed for any ROP.

Higher steroid intake was associated with lower cerebellar volume which is expected. The EGL in the cerebellum has the highest number of glucocorticoid receptors in the brain^{72,73}. Postnatal but not antenatal steroid exposure has been associated with impaired cerebellar growth⁷⁴.

In our study ROP was associated with reduced UWMV which is a reference to general white matter development at term equivalent age. Myelination is not apparent until at later stages²⁰³.

Clinical risk factors for developmental outcome

The mental developmental index (MDI) was associated with similar risk factors as any ROP and brain volumes *i.e.*, GA ($p = 0.001$), BW ($p = 0.004$), total steroid intake ($p = 0.006$) and additionally marginally associated with low Apgar score ($p = 0.047$).

Psychomotor developmental index (PDI) differed in its clinical risk factors and was associated with GA ($p = 0.015$) as any ROP and brain volumes but severe brain damage ($p = 0.025$) was an additional risk factor.

In our study any ROP exhibited a stronger association with reduced developmental quotients than did severe ROP. That applies to both mild impairment (< 85) and to moderate impairment (< 70). Table 3.

Table 3.

P value comparison of developmental quotients in infants with severe vs any ROP

	p value, severe ROP (Number of infants)	p value any ROP (number of infants)
MDI < 85	$p = 0.008$ (N=8)	$p < 0.001$ (N=14)
MDI < 70	$p = 0.02$ (N=5)	$p = 0.005$ (N=5)
PDI < 85	$p = 0.03$ (N=8)	$p = 0.02$ (N=14)
PDI < 70	$p = 0.11$ <i>ns</i> (N=3)	$p = 0.04$ (N=5)

Stages of ROP, brain volumes and developmental outcome

Infants with mild ROP had a lower mean UWMV and cerebellar volume ($p < 0.001$, $p < 0.001$) and lower developmental quotients, MDI and PDI ($p = 0.008$ and $p = 0.024$) as compared to infants without ROP.

Infants with treated ROP had lower UWMV, cerebellar volume and MDI ($p = 0.002$, $p = 0.005$, $p = 0.002$), whereas no significant difference could be shown for PDI, as compared to infants without ROP, Fig 11A-D.

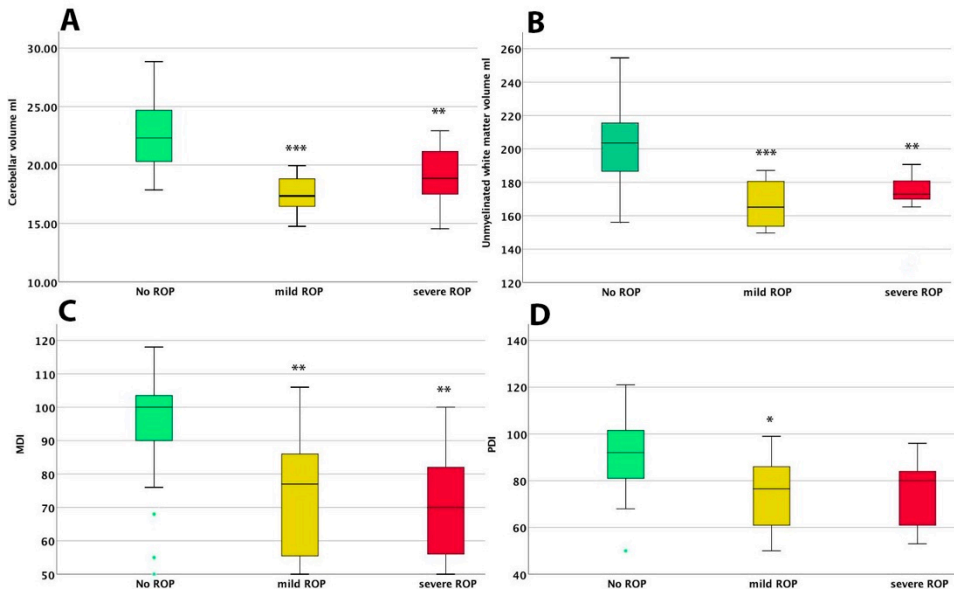


Fig 11. Stages of ROP in relation to brain volumes and developmental quotients.

Relationships between stages of ROP and **A** Cerebellar volume, **B** Unmyelinated white matter volume, **C** mental developmental index and **D** psychomotor developmental index. The different stages of ROP are defined as No ROP (green), mild ROP = stage 1 and 2, (yellow) and severe ROP = stage ≥ 3 (red). Boxplots denote medians and 25th and 75th percentiles.

After adjustment for GA, mean values of brain volumes and developmental outcome did not differ between infants with mild ROP and those with severe ROP, Fig 12 A-C

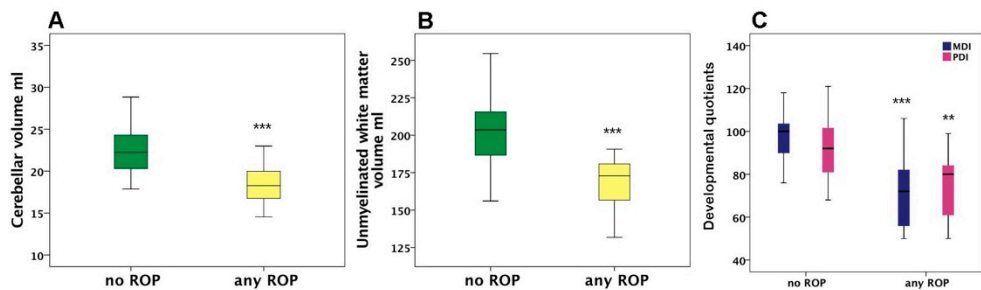


Fig 12. Cerebellar (A), unmyelinated white matter volume (B) and neurodevelopmental outcome (C) in infants with any ROP vs no ROP. Boxplots denote medians and 25th and 75th percentiles.

The associations between outcomes and any stage of ROP is not well studied. A very recent case-control study compared a small group (N=18) of preterm infants with severe ROP with infants with no ROP (N=36) and included infants with stage 1 ROP in the no ROP group. They found severe ROP to be associated with

reduced cerebellar and brainstem volumes at term and with early neurodevelopmental deficits at 15 months and 2 years of age¹⁴³. As in our study the mean GA was low, 25.3 weeks.

It is important to point out that the total number of included infants is small which reduces the power of the study. However, the incidence of treated ROP is high, the mild and severe groups were similar in size and the high rate of treatment in the severe group (9 out of 10 infants) has a potentially increasing effect on group differences.

Conclusions

The preterm rabbit pup model exhibits a combination of characteristics relevant to human preterm birth and cerebellar development. The preterm rabbit pup model is appropriate for use in future studies relating preterm birth to cerebellar development.

IVH in the preterm rabbit pup is followed by extensive deposition of cell-free Hb in cerebellar cell layers and white matter. The exposure to cell-free Hb was associated with microglial activation, an arrest in neuronal cell proliferation and delayed Purkinje cell maturation. Intraventricular administration of the cell-free Hb scavenger Hp partially blocked these effects suggesting that cell-free Hb and its downstream metabolites are causal in cerebellar impairment following IVH.

Development of any stage of ROP in very preterm infants was associated with reduced brain volumes and impaired developmental outcome at 2 years corrected age. Future studies addressing the association between ROP and neurodevelopment should consider the whole spectrum of ROP.

Enteral caffeine administration in preterm rabbit pups did not reveal any effects on growth or on the closely related trophic IGF-1 system in preterm rabbit pups and did not affect key neuronal maturation in the cerebellar external granular layer. It did increase survival.

General discussion and future perspectives

The main question of this thesis is why the cerebellum is smaller in preterm children compared to term born children and which neonatal events affect cerebellar development?

The general hypothesis is that preterm birth *per se* disturbs normal cerebellar development causing underdevelopment and that certain events following preterm birth, either complications or treatments, may aggravate the underdevelopment.

To improve the long-term outcome for preterm born infants a knowledge of damaging factors is crucial to be able to treat, or preferentially prevent adverse events that affect brain maturation. The conclusions drawn in this thesis are that key neuronal maturation is affected following preterm birth in rabbit pups and that IVH, with the subsequent distribution of the damaging cell-free Hb within the cerebellum, aggravates the damage. The damaging effect can be partially reversed with a cell-free Hb scavenger. The association between retinal and brain development suggests that factors reducing the risk of ROP could also reduce the risk of adverse brain development, including that of cerebellar development.

Retinopathy of prematurity, brain growth and developmental outcome in very preterm infants

Preterm birth coincides with a critical period of brain and vascular development²² which suggests the presence of common mechanisms in the development of these complications¹²³.

ROP is sometimes referred to as “the mirror of the brain”¹²⁴ and considered to be a spectrum of neurovascular disease. This theory is further supported by the observation that increasing severity of ROP correlates with a reduced thickness of retinal nerve fiber layer, assessed with spectral-domain optical coherence tomography in preterm born infants¹⁴². The retina is a part of the CNS and there is

a strong association between severe ROP and neurodevelopmental and/or visual impairment^{117,118}.

Preterm infants have low levels of serum IGF-1 from birth. These levels start to increase slowly around GW 30⁶⁴. The serum IGF-1 levels in our study cohort of preterm infants who developed ROP were significantly lower than those of preterm infants without ROP during the study period from GW 24 to 35. This is in agreement with reports from other studies that found low postnatal levels of IGF-1 to be strongly associated with development of ROP in preterm infants^{119,204}

Treatment-requiring ROP predicts white matter maturational delay at term equivalent age, independently of other signs of brain injury and is associated with lower developmental quotients at 18 months¹²². Factors reducing the risk of ROP could also reduce the risk of adverse brain development.

Almost all studies on ROP and outcome have focused on severe ROP, very few studies include the milder stages of ROP in their results. A possible explanation is that the milder stages usually regress within a few weeks and in that sense represent a “transient” state. Since ROP is a neurovascular disease we were interested to evaluate the association between the whole spectrum of ROP and brain volumes and developmental outcome. The number of infants treated for ROP was high in our study material, 17% compared to a mean incidence of 5.7% in Sweden¹²⁵. A possible explanation for the high incidence is that the mean (SD) GA was 26.4 (1.9) weeks and 25 of the infants were born below 26 gestational weeks. The large proportion of very immature infants may thus have increased the risk of treatment requiring ROP.

Future studies on ROP should include all stages of ROP. Finding ways to improve phase 1 of ROP would prevent the phase 2 and thereby reduce or even prevent ROP and improve brain growth and neurodevelopment. Improved nutritional status and thereby reduced postnatal growth restriction following preterm birth is one suggested method to reduce the risk of ROP.

A follow up at 2 years of age reflects early development and has limited prognostic value for later developmental impairment which indicates that longer follow-up time is needed for preterm infants.

Cell-free hemoglobin and cerebellar development

Cerebellar hypoplasia has repeatedly been shown to be associated with supratentorial IVH in preterm infants and to be a potential component in neurological disability^{25,49,60,61}. The severity of IVH is inversely correlated with cerebellar volume⁴⁸. We have shown that cell-free Hb has damaging effects in the

preterm cerebellum following IVH comparable to what has been shown in the choroid plexus and periventricular white matter following an IVH ^{102,104}.

In the cerebellum the most likely target exposed to cell-free Hb in the CSF would be the cells in the EGL that stand in direct interphase with the subarachnoidal space. This is consistent with our findings with a presence of cell-free Hb in the EGL and a reduction in EGL proliferation and a halted Purkinje cell maturation following IVH. We have also shown that cell-free Hb diffuses into the deeper layers, including the cerebellar white matter, indicating that all layers and thereby all celltypes can be affected and need to be considered in future studies.

Free iron has been found in the CSF for weeks after an IVH in preterm infants because of the limited ability of the CNS to discharge iron ¹¹⁰. The levels of endogenous Hb scavenger, Hp, are extremely low in both the circulation and in the brain of preterm infants ¹¹³. This endogenous defence system has therefore a very limited capacity to inactivate cell-free Hb ¹¹⁴. To be able to scavenge cell-free Hb following an IVH in the preterm infant exogenous Hp is needed and preferentially locally in the CSF and very likely soon after an IVH occurs. Being able to prevent the neurological damage following IVH could reduce the risk of long term complications in the smallest preterm infants.

Treatment with exogenous Hp is impossible today and there is a long road ahead. A treatment that could be given intravenously would be of preference to a treatment that needs to be administered directly into the CSF. Therefore, for Hp to be considered as a treatment for cell-free Hb scavenging following an IVH, studies are needed that include an evaluation of possible side effects, optimized timing and doses of administration for maximized scavenging. To date we have no administrative route of Hp into the ventricles of a newborn extremely preterm infant following an IVH. Are there other possible scavengers more appropriate or easier to use ?

Caffeine, the IGF-1 system and cerebellar development

No short time effects were observed with caffeine treatment in preterm rabbit pups. Administered caffeine had no effect on weight development, the IGF-1 system or on the development of the cerebellar external granular layer. The EGL development was only evaluated at one singular time-point. Downregulation of IGF-1 and the IGF-1R associated with caffeine exposure has been shown in different settings. We evaluated the endogenous IGF-1 system with a longitudinal evaluation of systemic IGF-1 including the liver as a prime source of circulating IGF-1 and found no impact on systemic levels of IGF-1. The evaluated dosage

would appear clinically relevant as resulting serum concentrations resemble therapeutic concentrations in preterm infants. Caffeine treatment had no effect on the quantitative evaluation of the IGF-1R. The highest density of IGF-1R was clearly localized to the Ki67 proliferative portion of the EGL with a distribution consisting with previous findings by others in postnatal rat brain ¹⁷⁹.

Caffeine treatment in preterm infants has been associated with a temporary growth restriction after starting treatment ¹⁵³. We saw no growth restriction with caffeine treatment in the preterm rabbit pups.

Caffeine did increase survival and the wet nurse model appears to have positive effects on postnatal growth and serum IGF-1 levels in preterm rabbit pups. Future studies should include evaluation of the cerebellar white matter development as well as a long-term follow-up. Thereafter, caffeine could be considered standard care for preterm rabbit pups to increase survival in future studies on cerebellar development.

Preterm birth *per se* and cerebellar development

Nutrition and postnatal growth

Severe growth restriction is well known in human infants following preterm birth with an initial growth restricted period until catch-up growth starts around GW 30 ^{64,65}. Nutrition and postnatal growth is associated with ROP development ¹³² and with head growth ¹²³. Head circumference is regarded as a proxy of brain growth and has been shown to be strongly associated with development of ROP ¹²³. Head circumference at term equivalent age is predictive of later developmental outcome ⁶⁶. Optimized nutritional intake may therefore be preventive of neurodevelopmental impairment.

In paper I, the preterm rabbit pups showed sign of undernutrition with absence of catch-up growth and a decrease in circulating IGF-1 levels. This effect was not seen in the preterm rabbit pups in paper IV who were fed by a wet nurse. This observation raises some questions about nutritional strategies. In the wet nurse model the pups receive the nutrition straight from the mother, they control their own feeding times and volumes and maybe receive some additional growth factors not provided by milk replacement formula.

The growth retardation phase in preterm infants is not eliminated by optimizing the protein and caloric intake alone, and the catch-up phase does not start until the low levels of serum IGF-1 start to increase ⁶⁴. This suggests that some additional

growth factors normally delivered to the fetus via the placenta are required to achieve growth comparable to normal growth *in utero* during the third trimester of pregnancy.

With this in mind it would be interesting to repeat the study in paper I with the wet nurse and evaluate the effect of preterm birth on cerebellar development under more optimal nutritional conditions and include a long-term follow-up with behavioural observations.

More studies on growth factor replacement are needed and the preterm rabbit pup model would be a suitable animal model for evaluation of the effects on both cerebellar and cerebral development.

Standardized methods for assessing the size of the cerebellum in the preterm rabbit pups are needed to include cerebellar growth measures in future studies.

External granular layer development

When studying cerebellar underdevelopment following preterm birth, the EGL is a logical start since the most important milestones of cerebellar development occur during the postnatal period following preterm birth. Proliferation declines earlier in preterm pups compared to term pups. Purkinje cell development is delayed both following preterm birth *per se* and following IVH. The observed changes following preterm birth *per se* might be sufficient to leave a permanent mark on cerebellar development. This pattern is seen in the weight development of preterm infants. Preterm infants experience growth retardation after birth and even though they catch up to normal weight at term equivalent age or within the first year the growth retardation leaves a long term mark with increased risk of ROP, reduced brain volumes and neurodevelopmental impairment. It is possible that the minor changes observed in cerebellar development are sufficient to cause disturbances that affect the cerebellum and its function as a control center in regulating movements, coordination and cognitive function in a long-term perspective.

As a final note, the cerebellum has a clear role in developmental impairment in preterm infants and deserves more attention both in the neonatal care of preterm infants and in research regarding development of preterm infants. Cerebellar imaging and evaluation should be included in the standard routine screening for brain abnormalities in the neonatal care of preterm infants. The results of the thesis support our general hypothesis and although it does not answer the main study question it provides some small steps towards the answer.

Svensk sammanfattning

Med förbättrad neonatalvård överlever fler och mera omogna barn idag. En del barn får bestående men på grund av sin prematuritet, där de minsta och mest omogna barnen löper störst risk. Cerebellum eller lillhjärnan är en del av hjärnan som deltar i att styra rörelser, koordination och kognition. Lillhjärnan innehåller fler nervceller än storhjärnan och är den delen av hjärnan som växer fortast under graviditetens sista trimester. På grund av sin snabba tillväxt är lillhjärnans utveckling speciellt känslig för de påfrestningar som en mycket prematur födelse kan innebära. Bildundersökningar med magnetröntgenteknik har visat att lillhjärnan hos mycket för tidigt födda barn är mindre när barnen uppnår fullgången tid jämfört med den hos barn som föds i fullgången tid. De senaste åren har problem relaterade till lillhjärnans utveckling uppmärksammas och lillhjärnans viktiga roll i utvecklingen blivit tydligare. Mekanismerna bakom hur lillhjärnans tillväxt hämmas är dock okända.

Huvudhypothesen i avhandlingen är att den prematura födelsen är huvudorsaken till lillhjärnans avvikande utveckling och att andra händelser under neonatalperioden som påverkar hjärnutvecklingen så som hjärnblödningar, prematuritetsretinopati eller möjligen mediciner som används kan bidra till ytterligare skada.

Prematurfödda kaniner har en hjärnutveckling som på många sätt liknar den som är känd hos prematura barn. Med denna djurmodell har vi kunnat visa att för tidig födelse leder till sämre tillväxt, lägre nivåer av en viktig tillväxtfaktor, insulin-like growth factor 1 (IGF-1), och att utmognaden av nyckelceller i lillhjärnan påverkas negativt. Vi visar att när röda blodkroppar bryts ner efter en hjärnblödning hos prematurfödda kaniner, tar sig fritt hemoglobin via ryggmärgsvätskan eller via nervbanor till lillhjärnans vävnad och orsakar underutveckling av lillhjärnan. I kaniner som fick en kroppseget protein, haptoglobin, insprutat i hjärnans hålrum blev skadeutvecklingen i lillhjärnan lindrigare. Proteinet haptoglobin binder starkt till det fria hemoglobinet och har därmed en oskadliggörande effekt. Dessa resultat ger hopp om att det i framtiden ska vara möjligt att minska skadan efter hjärnblödning hos för tidigt födda barn även om det är en lång väg dit. I tredje delarbetet undersökte vi effekten av koffein på tillväxt, IGF-1 och lillhjärnans utveckling hos prematurfödda kaniner. Koffein är ett av de mest använda läkemedlen i neonatalvården idag och ges för att förbättra andningsfunktionen hos

för tidigt födda barn. Koffeinets effekt på hjärnutvecklingen hos för tidigt födda barn är oklar och det finns tidigare data som talar för både positiva och negativa effekter. Hos prematurfödda kaninungar hade koffein ingen effekt på vare sig tillväxt, IGF-1 systemet eller på lillhjärnans utveckling. Som ett bifynd fann vi ökad överlevnad hos koffeinbehandlade kaninungar. Sista delarbetet handlar om prematurfödda barn som får prematuritetsretinopati eller retinopathy of prematurity (ROP). Prematuritetsretinopati är en sjukdom i kärl och nerver i ögats näthinna som kan leda till blindhet. Utveckling av en svår ROP hos prematurfödda barn sammanfaller ofta med andra problem inom barnets övriga psykomotoriska utveckling. I vår studie av mycket prematurfödda barn visar vi att även lindrigare stadier av ROP är kopplade till både minskade hjärnvolymer samt till en nedsatt psykomotorisk utveckling vid 2 års ålder.

För att förbättra långtidsprognosen för prematurfödda barn behövs mer kunskap om vad som orsakar hjärnskada för att kunna förhindra eller minska risken för bestående men.

Slutsatsen är att nervceller med nyckelfunktioner i lillhjärnan påverkas av mycket prematur födelse hos kaninungar och att hjärnblödning förvärrar lillhjärnans underutveckling. Underutvecklingen av lillhjärnan som följer hjärnblödningen kan minskas genom att tillföra kroppseget protein som blockerar de skadliga effekterna av fritt hemoglobin. Fortsatta studier behöver utvärdera tidpunkt och tillvägagångssätt för att, i framtiden, på säkert sätt kunna genomföra behandlingen hos mycket prematurfödda barn. Behandling med koffein hos prematurfödda kaniner visade sig inte påverka utvecklingen av lillhjärnan, IGF-1 systemet eller tillväxten. Det är lovande eftersom koffein är en så frekvent använd medicin i dagens neonatalvård. Kopplingen mellan utveckling av ROP och nedsatt hjärnutveckling, inklusive lillhjärnan, talar för närvaro av gemensamma bakomliggande riskfaktorer. Åtgärder för att påverka ROP skulle därmed kunna förbättra hjärnans tillväxt. Det finns studier som talar för att ett förbättrat nutritionsintag skulle kunna vara ett sätt att uppnå det målet.

Íslensk samantekt

Með framförum síðustu ára í umönnun minnstu fyrirburanna hafa lífslíkur þeirra aukist og mörk þess lífvænlega færst neðar. Þroskaskerðing hjá þessum hópi barna er algeng og líkurnar aukast eftir því sem börnin eru minni og óþroskaðri við fæðingu. Litli heilinn er mikilvægur hluti heilans og hefur hlutverk við stjórn hreyfinga, samhæfingar og vitsmunabroska. Í litla heilanum eru fleiri taugafrumur en í stóra heilanum og litli heilinn er sá hluti heilans sem vex örast á síðasta þriðjungi meðgöngunnar. Hann er því viðkvæmari en ella fyrir breytingum í kjölfar fyrirburafæðingar. Segulómskoðun hefur sýnt fram á að litli heilinn í miklum fyrirburum er minni við áætlaðan fæðingardag en litli heili fullmeðgenginna barna. Á undanförunum árum hefur þekking á þroskaröskunum sem tengjast litla heila aukist og mikilvægi litla heilans orðið skýrara. Orsakir á truflunum á þroska litla heilans eru hins vegar óþekktar.

Megintilgáta doktorsverkefnisins er sú að fæðing fyrir tímann sé meginástæða truflunar á þroska litla heilans og að aðrir fylgikvillar fyrirburafæðinga eins og heilablæðingar, sjónukvilli fyrirbura eða jafnvel lyf sem gefin eru fyrirburum auki neikvæð áhrif fyrirburafæðingar á þroska litla heilans.

Heilinn hjá kanínuungum sem eru fæddir fyrir tímann þroskast á svipaðan hátt og heili barna sem eru fædd fyrir tímann og því henta kanínuungar vel til rannsókna á áhrifum fyrirburafæðingar á þroska heilans. Þannig höfum við sýnt fram á að fæðing fyrir tímann veldur hægari vexti, skorti á mikilvægum vaxtarþáttum eins og Insulin-like growth factor 1 (IGF-1) og að lykilfrumur í litla heilanum þroskast hægar. Við höfum einnig sýnt fram á að í kjölfar heilablæðingar í kanínuungum sem fæddir eru fyrir tímann komast niðurbrotsafleiður blóðrauða með heila- og mænuvökvanum og/eða meðfram taugabrautum frá stóra heilanum til litla heilans. Þessi niðurbrotsefni valda síðan truflun í þroska á taugafrumum litla heilans. Prótein sem heitir haptoglobin hlutleysir hin skaðlegu niðurbrotsefni blóðrauða. Við sprautuðum haptoglobini inn í heila- og mænuvökva og gátum þannig minnkað verulega skaðleg áhrif blóðrauða á frumur litla heilans í kjölfar heilablæðingar. Þó svo að langt sé í land gefa þessar niðurstöður von um að það verði í framtíðinni hægt að minnka heilaskaða af völdum heilablæðinga í fyrirburum.

Við rannsökuðum áhrif koffeins á þyngdaraukningu, vaxtarþætti (IGF-1) og þroska litla heilans í kanínuungum sem fæddir voru fyrir tímann. Koffein er eitt

algengasta lyf sem notað er á nýburadeildum og bætir öndunarhæfni fyrirbura. Áhrif koffeins á heila fyrirbura eru ekki að fullu þekkt og fyrri niðurstöður hafa ýmist bent til jákvæðra eða neikvæðra áhrifa á hinn óþroskaða heila. Engin neikvæð áhrif koffeins sást á vöxt, vaxtarþætti eða á þroska litla heilans hjá kanínungunum. Hins vegar jókst lifun hjá þeim kaninum sem fengu koffein samanborið við kanínur sem fengu lyfleysu.

Síðasta verkefnið í doktorsritgerðinni fjallar um fylgni sjónukvilla fyrirbura, vaxtar heilans og taugaþroska við 2 ára aldur hjá hópi mikilla fyrirbura. Sjónukvilli fyrirbura er sjúkdómur í bæði æðum og taugum nethimnu augans sem getur leitt til blindu og sterk fylgni er á milli alvarlegs sjónukvilla og þroskaskerðingar. Við sýndum fram á fylgni ekki bara alvarlegs sjónukvilla heldur einnig vægs sjónukvilla við minni vöxt heilans, aðallega litla heilans og taugaþróaða heilans sem og við þroskaskerðingu við 2 ára aldur.

Þekking er lykilli framfara og til að bæta horfur minnstu fyrirburanna þarf að rannsaka orsakaþætti heilaskaða og þroskaskerðingar fyrirbura með það að markmiði að hindra eða meðhöndla áhættuþætti. Við getum minnkað neikvæð áhrif heilablæðingar á þroska litla heilans með próteininu haptoglobin sem hlutleysir skaðleg áhrif blóðrauða. Í framtíðinni þarf að rannsaka hvernig unnt er að gefa fyrirburum með heilablæðingu haptoglobin, kortleggja hugsanlegar aukaverkanir, tímasetningu meðferðar, skammtastærðir osfr. Meðferð með Koffeini hafði engin neikvæð áhrif á þroska litla heilans, vaxtarþáttinn IGF-1 eða þyngdaraukningu. Það er jákvætt þar sem koffein er eitt mest notaða lyfið í meðferð fyrirbura. Fylgni milli sjónukvilla fyrirbura og minni vaxtar heilans bendir til þess að um sömu áhættuþætti sé að ræða. Ef hægt er að minnka líkur fyrirbura á sjónukvilla er hugsanlegt að vöxtur heilans batni samhliða og einnig taugaþroski barnanna. Rannsóknir benda til þess að bætt næring minnstu fyrirburanna sé mikilvægur liður í því að bæta taugaþroska og rannsóknir eru nú þegar hafnar til að meta frekar áhrif næringar á horfur fyrirbura.

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References

1. Serenius F, Ewald U, Farooqi A, Fellman V, Hafström M, Maršál K, et al. Neurodevelopmental Outcomes Among Extremely Preterm Infants 6.5 Years After Active Perinatal Care in Sweden. *JAMA Pediatr* 2016;170:954-63.
2. Volpe JJ. The encephalopathy of prematurity-brain injury and impaired brain development inextricably intertwined. *Semin Pediatr Neurol* 2009;16:167-78.
3. Wood NS, Marlow N, Costeloe K, Gibson AT, Wilkinson AR. Neurologic and developmental disability after extremely preterm birth. EPICure Study Group. *N Engl J Med* 2000;343:378-84.
4. Marret S, Marchand-Martin L, Picaud JC, Hascoët JM, Arnaud C, Rozé JC, et al.; EPIPAGE Study Group. Brain injury in very preterm children and neurosensory and cognitive disabilities during childhood: The EPIPAGE Cohort Study. *PLoS One* 2013;8:e62683.
5. Serenius F, Källen K, Blennow M, Ewald U, Fellman V, Holmström G, et al.; EXPRESS Group. Neurodevelopmental outcome in extremely preterm infants at 2.5 years after active perinatal care in Sweden. *JAMA* 2013;309:1810-20.
6. Laptook AR, O'Shea TM, Shankaran S, Bhaskar B; NICHD Neonatal Network. Adverse neurodevelopmental outcomes among extremely low birth weight infants with a normal head ultrasound: prevalence and antecedents. *Pediatrics* 2005;115:673-80.
7. de Kieviet JF, Piek JP, Aarnoudse-Moens CS, Oosterlaan J. Motor development in very preterm and very low-birth-weight children from birth to adolescence: a meta-analysis. *JAMA* 2009;302:2235-42.
8. Aarnoudse-Moens CS, Weisglas-Kuperus N, van Goudoever JB, Oosterlaan J. Meta-analysis of neurobehavioral outcomes in very preterm and/or very low birth weight children. *Pediatrics* 2009;124:717-28.
9. Johnson S, Strauss V, Gilmore C, Jaekel J, Marlow N, Wolke D. Learning disabilities among extremely preterm children without neurosensory impairment: Comorbidity, neuropsychological profiles and scholastic outcomes. *Early Hum Dev* 2016;103:69-75.
10. Johnson S, Fawke J, Hennessy E, Rowell V, Thomas S, Wolke D, Marlow N. Neurodevelopmental disability through 11 years of age in children born before 26 weeks of gestation. *Pediatrics* 2009;124:249-57.
11. Farooqi A, Hägglöf B, Sedin G, Serenius F. Impact at age 11 years of major neonatal morbidities in children born extremely preterm. *Pediatrics* 2011;127:1247-57.
12. Volpe JJ. Cerebral white matter injury of the premature infant-more common than you think. *Pediatrics* 2003;112:176-80.

13. Schmahmann JD, Smith EE, Eichler FS, Filley CM. Cerebral white matter: neuroanatomy, clinical neurology, and neurobehavioral correlates. *Ann N Y Acad Sci* 2008;1142:266-309.
14. Dyet LE, Kennea N, Counsell SJ, Maalouf EF, Ajayi-Obe M, Duggan PJ, et al. Natural history of brain lesions in extremely preterm infants studied with serial magnetic resonance imaging from birth and neurodevelopmental assessment. *Pediatrics* 2006;118:536-48.
15. Inder TE, Warfield SK, Wang H, Hüppi PS, Volpe JJ. Abnormal cerebral structure is present at term in premature infants. *Pediatrics* 2005;115:286-94.
16. Peterson BS, Vohr B, Staib LH, Cannistraci CJ, Dolberg A, Schneider KC, et al. Regional brain volume abnormalities and long-term cognitive outcome in preterm infants. *JAMA* 2000;284:1939-47.
17. Peterson BS, Anderson AW, Ehrenkranz R, Staib LH, Tageldin M, Colson E, et al. Regional brain volumes and their later neurodevelopmental correlates in term and preterm infants. *Pediatrics* 2003;111:939-48.
18. Di Salvo DN. A new view of the neonatal brain: clinical utility of supplemental neurologic US imaging windows. *Radiographics* 2001;21:943-55.
19. Steggerda SJ, Leijser LM, Wiggers-de Bruine FT, van der Grond J, Walther FJ, van Wezel-Meijler G. Cerebellar injury in preterm infants: incidence and findings on US and MR images. *Radiology* 2009;252:190-9.
20. Parker J, Mitchell A, Kalpakidou A, Walshe M, Jung HY, Nosarti C, et al. Cerebellar growth and behavioural & neuropsychological outcome in preterm adolescents. *Brain* 2008;131:1344-51.
21. Limperopoulos C, Chilingaryan G, Sullivan N, Guizard N, Robertson RL, du Plessis AJ. Injury to the premature cerebellum: outcome is related to remote cortical development. *Cereb Cortex* 2014;24:728-36.
22. Volpe JJ. Cerebellum of the premature infant: rapidly developing, vulnerable, clinically important. *J Child Neurol* 2009;24:1085-104.
23. Brossard-Racine M, du Plessis AJ, Limperopoulos C. Developmental cerebellar cognitive affective syndrome in ex-preterm survivors following cerebellar injury. *Cerebellum* 2015;14:151-64.
24. Shah DK, Guinane C, August P, Austin NC, Woodward LJ, Thompson DK, et al. Reduced occipital regional volumes at term predict impaired visual function in early childhood in very low birth weight infants. *Invest Ophthalmol Visual Sci* 2006;47:3366-73.
25. Van Kooij BJ, Benders MJ, Anbeek P, Van Haastert IC, De Vries LS, Groenendaal F. Cerebellar volume and proton magnetic resonance spectroscopy at term, and neurodevelopment at 2 years of age in preterm infants. *Dev Med Child Neurol* 2012;54:260-6.
26. Bodensteiner JB, Johnsen SD. Magnetic resonance imaging (MRI) findings in children surviving extremely premature delivery and extremely low birthweight with cerebral palsy. *J Child Neurol* 2006;21:743-7.

27. Baumann O, Borra RJ, Bower JM, Cullen KE, Habas C, Ivry RB, et al. Consensus paper: the role of the cerebellum in perceptual processes. *Cerebellum* 2015;14:197-220.
28. E KH, Chen SH, Ho MH, Desmond JE. A meta-analysis of cerebellar contributions to higher cognition from PET and fMRI studies. *Hum Brain Mapp* 2014;35:593-615.
29. Allin M, Matsumoto H, Santhouse AM, Nosarti C, AlAsady MH, Stewart AL, et al. Cognitive and motor function and the size of the cerebellum in adolescents born very pre-term. *Brain* 2001;124:60–6.
30. Limperopoulos C, Bassan H, Gauvreau K, Robertson RL Jr, Sullivan NR, Benson CB, et al. Does cerebellar injury in premature infants contribute to the high prevalence of long-term cognitive, learning, and behavioral disability in survivors? *Pediatrics* 2007;120:584-93.
31. Limperopoulos C, Chilingaryan G, Sullivan N, Guizard N, Robertson RL, du Plessis AJ. Injury to the premature cerebellum: outcome is related to remote cortical development. *Cereb Cortex* 2014;24:728-36.
32. Reeber SL, Otis TS, Sillitoe RV. New roles for the cerebellum in health and disease. *Front Syst Neurosci* 2013;7:83.
33. Wang SS, Kloth AD, Badura A. The cerebellum, sensitive periods, and autism. *Neuron* 2014;83:518-32.
34. Liu Y, Xu D, Feng J, Kou H, Liang G, Yu H, et al. Fetal rat metabonome alteration by prenatal caffeine ingestion probably due to the increased circulatory glucocorticoid level and altered peripheral glucose and lipid metabolic pathways. *Toxicol Appl Pharmacol* 2012;262:205-16.
35. Schmahmann JD. Dysmetria of thought: clinical consequences of cerebellar dysfunction on cognition and affect. *Trends Cogn Sci* 1998;2:362-71.
36. Limperopoulos C, Soul JS, Gauvreau K, Hüppi PS, Warfield SK, Bassan H, et al. Late gestation cerebellar growth is rapid and impeded by premature birth. *Pediatrics* 2005;115:688-95.
37. Dahmane N, Ruiz i Altaba A. Sonic hedgehog regulates the growth and patterning of the cerebellum. *Development* 1999;126:3089-100.
38. Lewis PM, Gritli-Linde A, Smeyne R, Kottmann A, McMahon AP. Sonic hedgehog signaling is required for expansion of granule neuron precursors and patterning of the mouse cerebellum. *Dev Biol* 2004;270:393-410.
39. Ye P, Xing Y, Dai Z, D'Ercole AJ. In vivo actions of insulin-like growth factor-I (IGF-I) on cerebellum development in transgenic mice: evidence that IGF-I increases proliferation of granule cell progenitors. *Brain Res Dev Brain Res* 1996;95:44-54.
40. Biran V, Verney C, Ferriero DM. Perinatal cerebellar injury in human and animal models. *Neurol Res Int* 2012;2012:858929.
41. Sotelo C. Cellular and genetic regulation of the development of the cerebellar system. *Prog Neurobiol* 2004;72:295-339.
42. Goldowitz D, Hamre K. The cells and molecules that make a cerebellum. *Trends Neurosci* 1998;21:375-82.

43. De Luca A, Cerrato V, Fucá E, Parmigiani E, Buffo A, Leto K. Sonic hedgehog patterning during cerebellar development. *Cell Mol Life Sci* 2016;73:291-303.
44. Haldipur P, Bharti U, Govindan S, Sarkar C, Iyengar S, Gressens P, Mani S. Expression of Sonic hedgehog during cell proliferation in the human cerebellum. *Stem Cells Dev* 2012;21:1059-68.
45. Corrales JD, Blaess S, Mahoney EM, Joyner AL. The level of Sonic hedgehog signaling regulates the complexity of cerebellar foliation. *Development* 2006;133:1811-21.
46. Wang LC, Almazan G. Role of Sonic hedgehog signaling in oligodendrocyte differentiation. *Neurochem Res* 2016;41:3289-99.
47. Fernandez C, Tatard V, Bertrand N, Dahmane N. Differential modulation of Sonic-hedgehog- induced cerebellar granule Cell precursor proliferation by the IGF signaling network. *Dev Neurosci* 2010;32:59-70.
48. Tam EW, Miller SP, Studholme C, Chau V, Glidden D, Poskitt KJ, et al. Differential effects of intraventricular hemorrhage and white matter injury on preterm cerebellar growth. *J Pediatr* 2011;158:366-71.
49. Messerschmidt A, Prayer D, Brugger PC, Boltshauser E, Zoder G, Sterniste W, et al. Preterm birth and disruptive cerebellar development: assessment of perinatal risk factors. *Eur J of Paediatr Neurol* 2008;12:455-60.
50. Messerschmidt A, Brugger PC, Boltshauser E, Zoder G, Sterniste W, Birnbacher R, Prayer D. Disruption of cerebellar development: potential complication of extreme prematurity. *Am J Neuroradiol* 2005;26:1659-67.
51. Hansen-Pupp I, Hövel H, Hellström A, Hellström-Westas L, Löfqvist C, Larsson EM, et al. Postnatal decrease in circulating insulin-like growth factor-I and low brain volumes in very preterm infants. *J Clin Endocrinol Metab* 2011;96:1129-35.
52. Limperopoulos C, Soul JS, Haidar H, Hüppi PS, Bassan H, Warfield SK, et al. Impaired trophic interactions between the cerebellum and the cerebrum among preterm infants. *Pediatrics* 2005;116:844-50.
53. Tam EW, Ferriero DM, Xu D, Berman JI, Vigneron DB, Barkovich AJ, Miller SP. Cerebellar development in the preterm neonate: effect of supratentorial brain injury. *Pediatr Res* 2009;66:102-6.
54. Chua CO, Chahboune H, Braun A, Dummula K, Chua CE, Yu J, et al. Consequences of intraventricular hemorrhage in a rabbit pup model. *Stroke* 2009;40:3369-77.
55. Vinukonda G, Csiszar A, Hu F, Dummula K, Pandey NK, Zia MT, et al. Neuroprotection in a rabbit model of intraventricular haemorrhage by cyclooxygenase-2, prostanoid receptor-1 or tumour necrosis factor-alpha inhibition. *Brain* 2010;133:2264-80.
56. Hansen-Pupp I, Hövel H, Löfqvist C, Hellström-Westas L, Fellman V, Hüppi PS, et al. Circulatory insulin-like growth factor-I and brain volumes in relation to neurodevelopmental outcome in very preterm infants. *Pediatr Res* 2013;74:564-9.
57. Limperopoulos C, Benson CB, Bassan H, Disalvo DN, Kinnamon DD, Moore M, et al. Cerebellar hemorrhage in the preterm infant: ultrasonographic findings and risk factors. *Pediatrics* 2005;116:717-24.

58. Kitai Y, Hirai S, Ohmura K, Ogura K, Arai H. Cerebellar injury in preterm children with cerebral palsy after intraventricular hemorrhage: Prevalence and relationship to functional outcomes. *Brain Dev* 2015;37:758-63.
59. Tam EW, Rosenbluth G, Rogers EE, Ferriero DM, Glidden D, Goldstein RB, et al. Cerebellar hemorrhage on magnetic resonance imaging in preterm newborns associated with abnormal neurologic outcome. *J Pediatr* 2011;158:245-50.
60. Tam EW. Potential mechanisms of cerebellar hypoplasia in prematurity. *Neuroradiology* 2013;55:41-6.
61. Srinivasan L, Allsop J, Counsell SJ, Boardman JP, Edwards AD, Rutherford M. Smaller cerebellar volumes in very preterm infants at term-equivalent age are associated with the presence of supratentorial lesions. *Am J Neuroradiol* 2006;27:573-9.
62. Limperopoulos C, Chilingaryan G, Guizard N, Robertson RL, Du Plessis AJ. Cerebellar injury in the premature infant is associated with impaired growth of specific cerebral regions. *Pediatr Res* 2010;68:145-50.
63. Haldipur P, Bharti U, Alberti C, Sarkar C, Gulati G, Iyengar S, et al. Preterm delivery disrupts the developmental program of the cerebellum. *PLoS One* 2011;6:e233449.
64. Hansen-Pupp I, Löfqvist C, Polberger S, Niklasson A, Fellman V, Hellström A, Ley D. Influence of insulin-like growth factor I and nutrition during phases of postnatal growth in very preterm infants. *Pediatr Res* 2011;69:448-53.
65. Pilling EL, Elder CJ, Gibson AT. Growth patterns in the growth-retarded premature infant. *Best Pract Res Clin Endocrinol Metab* 2008;22:447-62.
66. Ehrenkranz RA, Dusick AM, Vohr BR, Wright LL, Wrage LA, Poole WK. Growth in the neonatal intensive care unit influences neurodevelopmental and growth outcomes of extremely low birth weight infants. *Pediatrics* 2006;117:1253-61.
67. Mallard C, Loeliger M, Copolov D, Rees S. Reduced number of neurons in the hippocampus and the cerebellum in the postnatal guinea-pig following intrauterine growth-restriction. *Neuroscience* 2000;100:327-33.
68. Tolsa CB, Zimine S, Warfield SK, Freschi M, Sancho Rossignol A, Lazeyras F, et al. Early alteration of structural and functional brain development in premature infants born with intrauterine growth restriction. *Pediatr Res* 2004;56:132-8.
69. Hutton LC, Yan E, Yawno T, Castillo-Melendez M, Hirst JJ, Walker DW. Injury of the developing cerebellum: a brief review of the effects of endotoxin and asphyxial challenges in the late gestation sheep fetus. *Cerebellum* 2014;13:777-86.
70. Biran V, Heine VM, Verney C, Sheldon RA, Spadafora R, Vexler ZS, et al. Cerebellar abnormalities following hypoxia alone compared to hypoxic-ischemic forebrain injury in the developing rat brain. *Neurobiol Dis* 2011;41:138-46.
71. Scheuer T, Sharkovska Y, Tarabykin V, Marggraf K, Brockmöller V, Bührer C, et al. Neonatal hyperoxia perturbs neuronal development in the cerebellum. *Mol Neurobiol* 2018;55:3901-15.
72. Pavlik A, Buresová M. The neonatal cerebellum: the highest level of glucocorticoid receptors in the brain. *Brain Res* 1984;314:13-20.

73. Noguchi KK, Walls KC, Wozniak DF, Olney JW, Roth KA, Farber NB. Acute neonatal glucocorticoid exposure produces selective and rapid cerebellar neural progenitor cell apoptotic death. *Cell Death Differ* 2008;15:1582–92.
74. Tam EW, Chau V, Ferriero DM, Barkovich AJ, Poskitt KJ, Studholme C, et al. Preterm cerebellar growth impairment after postnatal exposure to glucocorticoids. *Sci Transl Med* 2011;3:105ra105.
75. Heine VM, Rowitch DH. Hedgehog signaling has a protective effect in glucocorticoid-induced mouse neonatal brain injury through an 11betaHSD2-dependent mechanism. *J Clin Invest* 2009;119:267–77.
76. Croci L, Barili V, Chia D, Massimino L, van Vugt R, Masserdotti G, et al. Local Insulin-like growth factor I expression is essential for Purkinje neuron survival at birth. *Cell Death Differ* 2011;18:48–59.
77. Ishii N, Kono Y, Yonemoto N, Kusuda S, Fujimura M. Outcomes of infants born at 22 and 23 weeks gestation. *Pediatrics* 2013;132:62–71.
78. Stoll BJ, Hansen NI, Bell EF, Shankaran S, Laptook AR, Walsh MC, et al. Neonatal outcomes of extremely preterm infants from the NICHD Neonatal Research Network. *Pediatrics* 2010;126:443–56.
79. Wilson-Costello D, Friedman H, Minich N, Fanaroff AA, Hack M. Improved survival rates with increased neurodevelopmental disability for extremely low birth weight infants in the 1990s. *Pediatrics* 2005;115:997–1003.
80. Whitelaw A. Intraventricular haemorrhage and posthaemorrhagic hydrocephalus: pathogenesis, prevention and future interventions. *Semin Neonatol* 2001;6:135–46.
81. Ballabh P. Intraventricular hemorrhage in premature infants: mechanism of disease. *Pediatr Res* 2010;67:1–8.
82. Braun A, Xu H, Hu F, Kocherlakota P, Siegel D, Chander P, et al. Paucity of pericytes in germinal matrix vasculature of premature infants. *J Neurosci* 2007;27:12012–24.
83. Gould SJ, Howard S. Glial differentiation in the germinal layer of fetal and preterm infant brain: an immunocytochemical study. *Pediatr Pathol* 1988;8:25–36.
84. Nakamura Y, Okudera T, Fukuda S, Hashimoto T. Germinal matrix hemorrhage of venous origin in preterm neonates. *Hum Pathol* 1990;21:1059–62.
85. Papile LA, Burstein J, Burstein R, Koffler H. Incidence and evolution of subependymal and intraventricular hemorrhage: a study of infants with birth weights less than 1,500 gm. *J Pediatr* 1978;92:529–34.
86. Brouwer A, Groenendaal F, van Haastert IL, Rademaker K, Hanlo P, de Vries L. Neurodevelopmental outcome of preterm infants with severe intraventricular hemorrhage and therapy for post-hemorrhagic ventricular dilatation. *J Pediatr* 2008;152:648–54.
87. Murphy BP, Inder TE, Rooks V, Taylor GA, Anderson NJ, Mogridge N, et al. Posthaemorrhagic ventricular dilatation in the premature infant: natural history and predictors of outcome. *Arch Dis Child Fetal Neonatal Ed* 2002;87:F37–41.

88. Luu TM, Ment LR, Schneider KC, Katz KH, Allan WC, Vohr BR. Lasting effects of preterm birth and neonatal brain hemorrhage at 12 years of age. *Pediatrics* 2009;123:1037-44.
89. Reubsæet P, Brouwer AJ, van Haastert IC, Brouwer MJ, Koopman C, Groenendaal F, de Vries LS. The Impact of Low-Grade Germinal Matrix-Intraventricular Hemorrhage on Neurodevelopmental Outcome of Very Preterm Infants. *Neonatology* 2017;112:203-10.
90. Futagi Y, Toribe Y, Ogawa K, Suzuki Y. Neurodevelopmental outcome in children with intraventricular hemorrhage. *Pediatr Neurol* 2006;34:219-24.
91. Bolisetty S, Dhawan A, Abdel-Latif M, Bajuk B, Stack J, Lui K. Intraventricular hemorrhage and neurodevelopmental outcomes in extreme preterm infants. *Pediatrics* 2014;133:55-62.
92. Klebermass-Schrehof K, Czaba C, Olischar M, Fuiko R, Waldhoer T, Rona Z, et al. Impact of low-grade intraventricular hemorrhage on long-term neurodevelopmental outcome in preterm infants. *Childs Nerv Syst* 2012;28:2085-92.
93. Bourgouin PM, Tampieri D, Melancon D, del Carpio R, Ethier R. Superficial siderosis of the brain following unexplained subarachnoid hemorrhage: MRI diagnosis and clinical significance. *Neuroradiology* 1992;34:407-10.
94. Soto-Ares G, Vinchon M, Delmaire C, Abecidan E, Dhellemes P, Pruvo JP. Cerebellar atrophy after severe traumatic head injury in children. *Childs Nerv Syst* 2001;17:263-9.
95. Fukumizu M, Takashima S, Becker LE. Neonatal posthemorrhagic hydrocephalus: neuropathologic and immunohistochemical studies. *Pediatr Neurol* 1995;13:230-4.
96. Fukumizu M, Takashima S, Becker LE. Glial reaction in periventricular areas of the brainstem in fetal and neonatal posthemorrhagic hydrocephalus and congenital hydrocephalus. *Brain Dev* 1996;18:40-5.
97. Kumar S, Bandyopadhyay U. Free heme toxicity and its detoxification systems in human. *Toxicol Lett* 2005;157:175-88.
98. Quaye IK. Extracellular hemoglobin: the case of a friend turned foe. *Front Physiol* 2015;6:96.
99. Olsson MG, Allhorn M, Bülow L, Hansson SR, Ley D, Olsson ML, et al. Pathological conditions involving extracellular hemoglobin: molecular mechanisms, clinical significance, and novel therapeutic opportunities for alpha(1)-microglobulin. *Antioxid Redox Signal* 2012;17:813-46.
100. Khaket TP, Ahmad R. Biochemical studies on hemoglobin modified with reactive oxygen species (ROS). *Appl Biochem Biotechnol* 2011;164:1422-30.
101. Gram M, Sveinsdottir S, Ruscher K, Hansson SR, Cinthio M, Åkerström B, Ley D. Hemoglobin induces inflammation after preterm intraventricular hemorrhage by methemoglobin formation. *J Neuroinflammation* 2013;10:100.
102. Gram M, Sveinsdottir S, Cinthio M, Sveinsdottir K, Hansson SR, Mörgelin M, et al. Extracellular hemoglobin - mediator of inflammation and cell death in the choroid plexus following preterm intraventricular hemorrhage. *J Neuroinflammation* 2014;11:200.

103. Sveinsdottir S, Gram M, Cinthio M, Sveinsdottir K, Mörgelin M, Ley D. Altered expression of aquaporin 1 and 5 in the choroid plexus following preterm intraventricular hemorrhage. *Dev Neurosci* 2014;36:542-51.
104. Ley D, Romantsik O, Vallius S, Sveinsdottir S, Sveinsdottir K, Agyemang AA, et al. High presence of extracellular hemoglobin in the periventricular white matter following preterm intraventricular hemorrhage. *Front Physiol* 2016;7:330.
105. Nosarti C, Giouroukou E, Micali N, Rifkin L, Morris RG, Murray RM. Impaired executive functioning in young adults born very preterm. *J Int Neuropsychol Soc* 2007;13:571-81.
106. Lee JY, Keep RF, He Y, Sagher O, Hua Y, Xi G. Hemoglobin and iron handling in brain after subarachnoid hemorrhage and the effect of deferoxamine on early brain injury. *J Cereb Blood Flow Metab* 2010;30:1793-803.
107. Lok J, Leung W, Murphy S, Butler W, Noviski N, Lo EH. Intracranial hemorrhage: Mechanisms of secondary brain injury. *Acta Neurochir Suppl* 2011;111:63-9.
108. Indredavik MS, Vik T, Evensen KA, Skranes J, Taraldsen G, Brubakk AM. Perinatal risk and psychiatric outcome in adolescents born preterm with very low birth weight or term small for gestational age. *J Dev Behav Pediatr* 2010;31:286-94.
109. Gram M, Sveinsdottir S, Ruscher K, Hansson SR, Cinthio M, Åkerström B, Ley D. Hemoglobin induces inflammation after preterm intraventricular hemorrhage by methemoglobin formation. *J Neuroinflammation* 2013;10:100.
110. Savman K, Nilsson UA, Blennow M, Kjellmer I, Whitelaw A. Non-protein-bound iron is elevated in cerebrospinal fluid from preterm infants with posthemorrhagic ventricular dilatation. *Pediatr Res* 2001;49:208-12.
111. Abraham NG, Drummond G. CD163-Mediated hemoglobin-heme uptake activates macrophage HO-1, providing an antiinflammatory function. *Circ Res* 2006;99:911-4.
112. Chintagari NR, Nguyen J, Belcher JD, Vercellotti GM, Alayash AI. Haptoglobin attenuates hemoglobin-induced heme oxygenase-1 in renal proximal tubule cells and kidneys of a mouse model of sickle cell disease. *Blood cells Mol Dis* 2015;54:302-6.
113. Chavez-Bueno S, Beasley JA, Goldbeck JM, Bright BC, Morton DJ, Whitby PW, Stull TL. Haptoglobin concentrations in preterm and term newborns. *J Perinatol* 2011;31:500-3.
114. Galea J, Cruickshank G, Teeling JL, Boche D, Garland P, Perry VH, Galea I. The intrathecal CD163-haptoglobin-hemoglobin scavenging system in subarachnoid hemorrhage. *J Neurochem* 2012;121:785-92.
115. Hellgren KM, Törnqvist K, Jakobsson PG, Lundgren P, Carlsson B, Källén K, et al. Ophthalmologic Outcome of Extremely Preterm Infants at 6.5 Years of Age: Extremely Preterm Infants in Sweden Study (EXPRESS). *JAMA Ophthalmol* 2016;134:555-562.
116. Allred EN, Capone A Jr, Fraioli A, Dammann O, Droste P, Duker J, et al. Retinopathy of prematurity and brain damage in the very preterm newborn. *J AAPOS* 2014;18:241-7.
117. Hellström A, Smith LE, Dammann O. Retinopathy of prematurity. *Lancet* 2013;381:1445-57.

118. Chen J, Smith LE. Retinopathy of prematurity. *Angiogenesis* 2007;10:133-40.
119. Hellström A, Engström E, Hård AL, Albertsson-Wikland K, Carlsson B, Niklasson A, et al. Postnatal serum insulin-like growth factor I deficiency is associated with retinopathy of prematurity and other complications of premature birth. *Pediatrics* 2003;112:1016-20.
120. Beligere N, Perumalswamy V, Tandon M, Mittal A, Floora J, Vijayakumar B, Miller MT. Retinopathy of prematurity and neurodevelopmental disabilities in premature infants. *Semin Fetal Neonatal Med* 2015;20:346-53.
121. Schmidt B, Davis PG, Asztalos EV, Solimano A, Roberts RS. Association between severe retinopathy of prematurity and nonvisual disabilities at age 5 years. *JAMA* 2014;311:523-5.
122. Glass TJA, Chau V, Gardiner J, Foong J, Vinal J, Zwicker JG, et al. Severe retinopathy of prematurity predicts delayed white matter maturation and poorer neurodevelopment. *Arch Dis Child Fetal Neonatal Ed* 2017;102:F532-37.
123. Löfqvist C, Engström E, Sigurdsson J, Hård AL, Niklasson A, Ewald U, et al. Postnatal head growth deficit among premature infants parallels retinopathy of prematurity and insulin-like growth factor-1 deficit. *Pediatrics* 2006;117:1930-8.
124. Msall ME. The retina as a window to the brain in vulnerable neonates. *Pediatrics* 2006;117:2287-9.
125. Holmström G, Törnqvist K, Al-Hawasi A, Nilsson Å, Wallin A, Hellström A. Increased frequency of retinopathy of prematurity over the last decade and significant regional differences. *Acta ophthalmol* 2018;96:142-8.
126. Prematurity ICfCoRo. The International Classification of Retinopathy of Prematurity revisited. *Arch Ophthalmol* 2005;123:991-9.
127. Darlow BA, Hutchinson J, Henderson-Smart DJ, Donoghue DA, Simpson JM, Evans NJ. Prenatal risk factors for severe retinopathy of prematurity among very preterm infants of the Australian and New Zealand Neonatal Network. *Pediatrics* 2005;115:990-6.
128. Lundgren P, Wilde Å, Löfqvist C, Smith LE, Hård AL, Hellström A. Weight at first detection of retinopathy of prematurity predicts disease severity. *Br J Ophthalmol* 2014;98:1565-9.
129. Lee JW, VanderVeen D, Allred EN, Leviton A, Dammann O. Prethreshold retinopathy in premature infants with intrauterine growth restriction. *Acta Paediatr* 2015;104:27-31.
130. Hellström A, Perruzzi C, Ju M, Engström E, Hård AL, Liu JL, et al. Low IGF-I suppresses VEGF-survival signaling in retinal endothelial cells: direct correlation with clinical retinopathy of prematurity. *Proc Natl Acad Sci U S A* 2001;98:5804-8.
131. Wu C, Vanderveen DK, Hellström A, Löfqvist C, Smith LE. Longitudinal postnatal weight measurements for the prediction of retinopathy of prematurity. *Arch Ophthalmol* 2010;128:443-7.
132. Stoltz Sjöström E, Lundgren P, Öhlund I, Holmström G, Hellström A, Domellöf M. Low energy intake during the first 4 weeks of life increases the risk for severe retinopathy of prematurity in extremely preterm infants. *Arch Dis Child Fetal Neonatal Ed* 2016;101:F108-F13.

133. Löfqvist CA, Najm S, Hellgren G, Engström E, Sävman K, Nilsson AK, et al. Association of Retinopathy of Prematurity With Low Levels of Arachidonic Acid: A Secondary Analysis of a Randomized Clinical Trial. *JAMA Ophthalmol* 2018;136:271-277.
134. Carlo WA, Finer NN, Walsh MC, Rich W, Laptook AR, Yoder BA, et al. Target ranges of oxygen saturation in extremely preterm infants. *N Engl J Med* 2010;362:1959-69.
135. Stenson B, Brocklehurst P, Tarnow-Mordi W. Increased 36-week survival with high oxygen saturation target in extremely preterm infants. *N Engl J Med* 2011;364:1680-2.
136. Chen ML, Guo L, Smith LE, Dammann CE, Dammann O. High or low oxygen saturation and severe retinopathy of prematurity: a meta-analysis. *Pediatrics* 2010;125:e1483-92.
137. Hellström A, Källen K, Carlsson B, Holmström G, Jakobsson P, Lundgren P, et al. Extreme prematurity, treated retinopathy, bronchopulmonary dysplasia and cerebral palsy are significant risk factors for ophthalmological abnormalities at 6.5 years of age. *Acta Paediatr* 2018.
138. Bassi L, Ricci D, Volzone A, Allsop JM, Srinivasan L, Pai A, et al. Probabilistic diffusion tractography of the optic radiations and visual function in preterm infants at term equivalent age. *Brain* 2008;131:573-82.
139. Kelly CE, Cheong JL, Molloy C, Anderson PJ, Lee KJ, Burnett AC, et al.; Victorian Infant Collaborative Study Group. Neural Correlates of Impaired Vision in Adolescents Born Extremely Preterm and/or Extremely Low Birthweight. *PLoS One* 2014;9:e93188.
140. Msall ME, Phelps DL, Hardy RJ, Dobson V, Quinn GE, Summers CG, Tremont MR; Cryotherapy for Retinopathy of Prematurity Cooperative Group. Educational and social competencies at 8 years in children with threshold retinopathy of prematurity in the CRYO-ROP multicenter study. *Pediatrics* 2004;113:790-9.
141. Msall ME, Phelps DL, DiGaudio KM, Dobson V, Tung B, McCleod RE, et al. Severity of neonatal retinopathy of prematurity is predictive of neurodevelopmental functional outcome at age 5.5 years. *Pediatrics* 2000;106:998-1005.
142. Park KA, Oh SY. Retinal nerve fiber layer thickness in prematurity is correlated with stage of retinopathy of prematurity. *Eye (Lond)* 2015;12:1594-602.
143. Drost FJ, Keunen K, Moeskops P, Claessens NHP, van Kalken F, Išgum I, et al. Severe retinopathy of prematurity is associated with reduced cerebellar and brainstem volumes at term and neurodevelopmental deficits at 2 years. *Pediatr Res* 2018.
144. Hsieh EM, Hornik C, Clark RH, Laughon MM, Benjamin DK Jr, Smith PB; Best Pharmaceuticals for Children Act—Pediatric Trials Network. Medication use in the neonatal intensive care unit. *Am J Perinatol* 2014;31:811-21.
145. Henderson-Smart DJ, de Paoli AG. Methylxanthine treatment for apnoea in preterm infants. *Cochrane Database of Systematic Reviews* 2010.
146. Lachance MP, Marlowe C, Waddell WJ. Autoradiographic disposition of [1-methyl-14C]- and [2-14C]caffeine in mice. *Toxicol Appl Pharmacol* 1983;71:237-41.

147. Atik A, Harding R, De Matteo R, Kondos-Devic D, Cheong J, Doyle LW, Tolcos M. Caffeine for apnea of prematurity: Effects on the developing brain. *Neurotoxicology* 2017;58:94-102.
148. Millar D, Schmidt B. Controversies surrounding xanthine therapy. *Semin Neonatol* 2004;9:239-44.
149. Dunwiddie TV, Masino SA. The role and regulation of adenosine in the central nervous system. *Annu Rev Neurosci* 2001;24:31.
150. Rudolphi KA, Schubert P, Parkinson FE, Fredholm BB. Neuroprotective role of adenosine in cerebral ischaemia. *Trends Pharmacol Sci* 1992;13:439-45.
151. Bona E, Ådén U, Gilland E, Fredholm BB, Hagberg H. Neonatal cerebral hypoxia-ischemia: the effect of adenosine receptor antagonists. *Neuropharmacology* 1997;36:1327-38.
152. Ådén U, Halldner L, Lagercrantz H, Dalmau I, Ledent C, Fredholm BB. Aggravated brain damage after hypoxic ischemia in immature adenosine A2A knockout mice. *Stroke* 2003;34:739-44.
153. Schmidt B, Roberts RS, Davis P, Doyle LW, Barrington KJ, Ohlsson A, et al. Caffeine therapy for apnea of prematurity. *N Engl J Med* 2006;354:2112-21.
154. Schmidt B, Roberts RS, Davis P, Doyle LW, Barrington KJ, Ohlsson A, et al. Long-term effects of caffeine therapy for apnea of prematurity. *N Eng J Med* 2007;357:1893-902.
155. Schmidt B, Anderson PJ, Doyle LW, Dewey D, Grunau RE, Asztalos EV, et al. Survival without disability to age 5 years after neonatal caffeine therapy for apnea of prematurity. *JAMA* 2012;307:275-82.
156. Crossley KJ, Allison BJ, Polglase GR, Morley CJ, Harding R, Davis PG, et al. Effects of caffeine on renal and pulmonary function in preterm newborn lambs. *Pediatr Res* 2012;72:19-25.
157. Bauer J, Maier K, Linderkamp O, Hentschel R. Effect of caffeine on oxygen consumption and metabolic rate in very low birth weight infants with idiopathic apnea. *Pediatrics* 2001;107:660-3.
158. Winerdal M, Urmaliya V, Winerdal ME, Fredholm BB, Winqvist O, Ådén U. Single Dose Caffeine Protects the Neonatal Mouse Brain against Hypoxia Ischemia. *Plos One* 2017;12:e0170545.
159. Bona E, Ådén U, Fredholm BB, Hagberg H. The effect of long term caffeine treatment on hypoxic-ischemic brain damage in the neonate. *Pediatr Res* 1995;38:312-8.
160. Yazdani M, Hartman AD, Miller HI, Temples TE, Nakamoto T. Chronic caffeine intake alters the composition of various parts of the brain in young growing rats. *Dev Pharmacol Ther* 1988;11:102-8.
161. Yazdani M, Ide K, Asadifar M, Gottschalk S, Joseph F Jr, Nakamoto T. Effects of caffeine on the saturated and monounsaturated fatty acids of the newborn rat cerebellum. *Ann Nutr Metab* 2004;48:79-83.
162. Crespo M, León-Navarro DA, Martin M. Cerebellar oxidative stress and fine motor impairment in adolescent rats exposed to hyperthermia-induced seizures is prevented

- by maternal caffeine intake during gestation and lactation. *Eur J Pharmacol* 2018;822:186-98.
163. McPherson C, Neil JJ, Tjoeng TH, Pineda R, Inder TE. A pilot randomized trial of high-dose caffeine therapy in preterm infants. *Pediatr Res* 2015;78:198-204.
 164. Etzel BA, Guillet R. Effects of neonatal exposure to caffeine on adenosine A1 receptor ontogeny using autoradiography. *Brain Res Dev Brain Res* 1994;82:223-30.
 165. Aranda JV, Cai CL, Ahmad T, Bronshtein V, Valencia GB, Lazzaro DR, Beharry KD. Pharmacologic synergism of ocular ketorolac and systemic caffeine citrate in rat oxygen-induced retinopathy. *Pediatr Res* 2016;80:554-65.
 166. Langford K, Nicolaides K, Miell JP. Maternal and fetal insulin-like growth factors and their binding proteins in the second and third trimesters of human pregnancy. *Hum Reprod* 1998;13:1389-93.
 167. Laviola L, Natalicchio A, Perrini S, Giorgino F. Abnormalities of IGF-I signaling in the pathogenesis of diseases of the bone, brain, and fetoplacental unit in humans. *Am J Physiol Endocrinol Metab* 2008;295:E991-9.
 168. Cheng CM, Reinhardt RR, Lee WH, Joncas G, Patel SC, Bondy CA. Insulin-like growth factor 1 regulates developing brain glucose metabolism. *Proc Natl Acad Sci U S A* 2000;97:10236-41.
 169. Johansson PA, Dziegielewska KM, Liddelow SA, Saunders NR. The blood-CSF barrier explained: when development is not immaturity. *Bioessays* 2008;30:237-48.
 170. Reinhardt RR, Bondy CA. Insulin-like growth factors cross the blood-brain barrier. *Endocrinology* 1994;135:1753-61.
 171. Roberts CT, Owens JA, Carter AM, Harding JE, Austgulen R, Wlodek M. Insulin-like growth factors and foetal programming—A workshop report. *Placenta* 2003;Suppl A:S72-5.
 172. Hellström A, Ley D, Hansen-Pupp I, Hallberg B, Löfqvist C, van Marter L, et al. Insulin-like growth factor 1 has multisystem effects on foetal and preterm infant development. *Acta Paediatr* 2016;105:576-86.
 173. Oliver MH, Harding JE, Breier BH, Gluckman PD. Fetal insulin-like growth factor (IGF)-I and IGF-II are regulated differently by glucose or insulin in the sheep fetus. *Reprod Fertil Dev* 1996;8:167-72.
 174. Baumann MU, Schneider H, Malek A, Palta V, Surbek DV, Sager R, et al. Regulation of human trophoblast GLUT1 glucose transporter by insulin-like growth factor I (IGF-I). *PLoS One* 2014;9:e106037.
 175. Woods KA, Camacho-Hübner C, Savage MO, Clark AJ. Intrauterine growth retardation and postnatal growth failure associated with deletion of the insulin-like growth factor I gene. *N Engl J Med* 1996;335:1363-7.
 176. Fryklund L, Gluckman P, Skottner A. Treatment of catabolic states using authentic IGF-I and hypocaloric amount of nutrients. US Patent # 6034059 <http://patentscom/us-6034059.html>.
 177. Jensen AK, Ying GS, Huang J, Quinn GE, Binenbaum G. Postnatal serum Insulin-like growth factor I and retinopathy of prematurity. *Retina* 2017;37:867-72.

178. Lee WH, Javedan S, Bondy CA. Coordinate Expression of Insulin-like growth factor system components by neurons and neuroglia during retinal and cerebellar development. *J Neurosci* 1992;12:4737-44.
179. Bondy C, Werner H, Roberts CT Jr, LeRoith D. Cellular pattern of type-I insulin-like growth factor receptor gene expression during maturation of the rat brain: comparison with insulin-like growth factors I and II. *Neuroscience* 1992;46:909-23.
180. Carson MJ, Behringer RR, Brinster RL, McMorris FA. Insulin-like growth factor I increases brain growth and central nervous system myelination in transgenic mice. *Neuron* 1993;10:729-40.
181. Chrysis D, Calikoglu AS, Ye P, D'Ercole AJ. Insulin-like growth factor-I overexpression attenuates cerebellar apoptosis by altering the expression of Bcl family proteins in a developmentally specific manner. *J Neurosci* 2001;21:1481-9.
182. O'Kusky JR, Ye P, D'Ercole AJ. Insulin-like growth factor-I promotes neurogenesis and synaptogenesis in the hippocampal dentate gyrus during postnatal development. *J Neurosci* 2000;20:8435-42.
183. Carro E, Nunez A, Busiguina S, Torres-Aleman I. Circulating insulin-like growth factor I mediates effects of exercise on the brain. *J Neurosci* 2000;20:2926-33.
184. Lin X, Bulleit RF. Insulin-like growth factor I (IGF-I) is a critical trophic factor for developing cerebellar granule cells. *Brain Res Dev Brain Res* 1997;99:234-42.
185. Torres-Aleman I, Villalba M, Nieto-Bona MP. Insulin-like growth factor-I modulation of cerebellar cell populations is developmentally stage-dependent and mediated by specific intracellular pathways. *Neuroscience* 1998;83:321-34.
186. Linseman DA, Phelps RA, Bouchard RJ, Le SS, Laessig TA, McClure ML, Heidenreich KA. Insulin-like growth factor-I blocks Bcl-2 interacting mediator of cell death (Bim) induction and intrinsic death signaling in cerebellar granule neurons. *J Neurosci* 2002;22:9287-97.
187. Kakizawa S, Yamada K, Iino M, Watanabe M, Kano M. Effects of insulin-like growth factor I on climbing fibre synapse elimination during cerebellar development. *Eur J Neurosci* 2003;17:545-54.
188. Jacobo SM, Kazlauskas A. Insulin-like growth factor 1 (IGF-1) stabilizes nascent blood vessels. *J Biol Chem* 2015;290:6349-60.
189. Tan Y, Liu J, Deng Y, Cao H, Xu D, Cu F, et al. Caffeine-induced fetal rat over-exposure to maternal glucocorticoid and histone methylation of liver IGF-1 might cause skeletal growth retardation. *Toxicol Lett* 2012;214:279-87.
190. Rosendahl AH, Perks CM, Zeng L, Markkula A, Simonsson M, Rose C, et al. Caffeine and caffeic acid inhibit growth and modify estrogen receptor and Insulin-like growth factor I receptor levels in human breast cancer. *Clin Cancer Res* 2015;21:1877-87.
191. Wang Y, Dakubo GD, Thurig S, Mazerolle CJ, Wallace VA. Retinal ganglion cell-derived sonic hedgehog locally controls proliferation and the timing of RGC development in the embryonic mouse retina. *Development* 2005;132:5103-13.
192. Steck J, Bluemi C, Kampmann S, Greene B, Maier RF, Arnhold S, et al. Retinal vessel pathologies in a rat model of periventricular leukomalacia: a new model for retinopathy of prematurity? *Invest Ophthalmol Vis Sci* 2015;56:1830-41.

193. Yuen TJ, Silbereis JC, Griveau A, Chang SM, Daneman R, Fancy SPJ, et al. Oligodendrocyte-encoded HIF function couples postnatal myelination and white matter angiogenesis. *Cell* 2014;158:383-396.
194. Lorenzo AV, Welch K, Conner S. Spontaneous germinal matrix and intraventricular hemorrhage in prematurely born rabbits. *J Neurosurg* 1982;56:404-10.
195. Clancy B, Kersh B, Hyde J, Darlington RB, Anand KJ, Finlay BL. Web-based method for translating neurodevelopment from laboratory species to humans. *Neuroinformatics* 2007;5:79-94.
196. Sveinsdottir S, Cinthio M, Ley D. High-frequency ultrasound in the evaluation of cerebral intraventricular haemorrhage in preterm rabbit pups. *Ultrasound Med Biol* 2012;38:423-31.
197. Marsal K, Persson PH, Larsen T, Lilja H, Selbing A, Sultan B. Intrauterine growth curves based on ultrasonically estimated foetal weights. *Acta Paediatr* 1996;85:843-8.
198. Group ETFROP. Revised indications for the treatment of retinopathy of prematurity: results of the early treatment for retinopathy of prematurity randomized trial. *Arch Ophthalmol* 2003;121:1684-94.
199. Traudt CM, McPherson RJ, Studholme C, Millen KJ, Juul SE. Systemic glycerol decreases neonatal rabbit brain and cerebellar growth independent of intraventricular hemorrhage. *Pediatr Res* 2014;75:389-94.
200. Andersson IK, Edwall D, Norstedt G, Rozell B, Skottner A, Hansson HA. Differing expression of insulin-like growth factor I in the developing and in the adult rat cerebellum. *Acta Physiol Scand* 1988;132:167-73.
201. Dummula K, Vinukonda G, Chu P, Xing Y, Hu F, Mailk S, et al. Bone morphogenetic protein inhibition promotes neurological recovery after intraventricular hemorrhage. *J Neurosci* 2011;31:12068-82.
202. Volpe JJ, Kinney HC, Jensen FE, Rosenberg PA. The developing oligodendrocyte: key cellular target in brain injury in the premature infant. *Int J Dev Neurosci* 2011;29:423-40.
203. Kinney HC, Brody BA, Kloman AS, Gilles FH. Sequence of central nervous system myelination in human infancy. II. Patterns of myelination in autopsied infants. *J Neuropathol Exp Neurol* 1988;47:217-34.
204. Perez-Munuzuri A, Fernandez-Lorenzo JR, Couce-Pico ML, Blanco-Teijeiro MJ, Fraga-Bermudez JM. Serum levels of IGF1 are a useful predictor of retinopathy of prematurity. *Acta Paediatr* 2010;99:519-25.

Paper I



Impaired Cerebellar Maturation, Growth Restriction, and Circulating Insulin-Like Growth Factor 1 in Preterm Rabbit Pups

Kristbjörg Sveinsdóttir^a John-Kalle Lämsberg^a Snjólaug Sveinsdóttir^a
Martin Garwicz^c Lennart Ohlsson^d Ann Hellström^e Lois Smith^f
Magnus Gram^b David Ley^a

Departments of ^aPediatrics and ^bInfection Medicine, ^cNeuronano Research Center, Lund University, and ^dMicromorph AB, Lund, ^eDepartment of Ophthalmology, Institute of Neuroscience and Physiology, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden; ^fDepartment of Ophthalmology, Boston Children's Hospital, Boston, MA, USA

Keywords

Preterm birth · Cerebellum · Insulin-like growth factor 1 · Purkinje cells · Sonic hedgehog protein

Abstract

Cerebellar growth is impeded following very preterm birth in human infants and the observed reduction in cerebellar volume is associated with neurodevelopmental impairment. Decreased levels of circulating insulin-like growth factor 1 (IGF-1) are associated with decreased cerebellar volume. The relationship between preterm birth, circulating IGF-1, and key cell populations supporting cerebellar proliferation is unknown. The aim of this study was to evaluate the effect of preterm birth on postnatal growth, circulating IGF-1, and cerebellar maturation in a preterm rabbit pup model. Preterm rabbit pups (PT) were delivered by cesarean section at day 29 of gestation, cared for in closed incubators with humidified air, and gavage fed with formula. Control term pups (T) delivered by spontaneous vaginal delivery at day 32 of gestation were housed and fed by their lactating doe. In vivo perfusion-fixation for immunohistochemical evaluation of cerebellar proliferation, cell maturation, and apoptosis was performed at repeated time points in PT and T pups. Results show that the mean weight of the pups and circulating IGF-1 protein levels were lower in the PT group at all time points ($p < 0.05$) than in the T group. Postnatal weight development correlated with circulating IGF-1 ($r^2 = 0.89$) independently of gestational age at birth and postnatal age. The proliferative (Ki-67-positive) portion of the external granular layer (EGL) was decreased in the PT group at postnatal day 2 (P2) compared to in the T group ($p = 0.01$). Purkinje cells exhibited decreased calbindin staining at P0 ($p = 0.003$), P2 ($p = 0.004$), and P5 ($p = 0.04$) in the PT group compared to in the T group. Staining for sonic hedgehog was positive in neuronal EGL progenitors and Purkinje cells at early time points but was restricted to a well-defined Purkinje cell monolayer at later time points. Preterm birth in rabbit pups is associated with lower circulating levels of IGF-1, decreased postnatal growth, and decreased cerebellar EGL proliferation and Purkinje cell maturation. The preterm rabbit pup model exhibits important characteristics of human preterm birth, and may thus be suitable for the evaluation of interventions aiming to modify growth and cerebellar development in the preterm population.

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E-Mail karger@karger.com
www.karger.com/dne

David Ley, MD, PhD
Lund University, Skane University Hospital, Department of Clinical Sciences, Pediatrics
BMC C14
SE-221 84 Lund (Sweden)
E-Mail david.ley@med.lu.se

Introduction

The improved survival of very preterm infants has increased the awareness that very preterm birth is associated with diverse neurodevelopmental disability [1–3]. The knowledge on brain development following preterm birth is insufficient. Acquired lesions such as intraventricular hemorrhage and white-matter damage of the cerebrum have well-defined implications for neurodevelopmental impairment. However, the mechanisms whereby very premature birth per se has a damaging effect on brain development remain less well defined.

The use of magnetic resonance imaging has increased awareness of cerebellar abnormalities following preterm birth and their important contribution to neurodevelopmental disability. In humans, the cerebellum is the fastest-growing part of the brain during late pregnancy. The cerebellar volume increases 5-fold and the surface area increases 30-fold during the 3rd trimester. Consequently, very premature birth may have important implications for the structural and functional integrity of the cerebellum.

In very preterm human infants, the cerebellar volume, as determined by magnetic resonance imaging, is smaller at term age than in control term infants [4, 5]. The observed reduction in cerebellar volume has been associated with acquired brain insults such as intraventricular hemorrhage [6, 7], but is also present in relation to extreme prematurity per se [4, 8]. Importantly, reduced cerebellar volume at term age has been associated with subsequent neurological impairment [6, 9, 10].

The mechanisms involved in reduced cerebellar volume following very preterm birth remain unknown. We have shown that very preterm birth is followed by decreased circulating levels of insulin-like growth factor 1 (IGF-1) [11]. Continued study showed that decreased levels of IGF-1 were associated with decreased brain volumes at term age, with the cerebellum exhibiting the strongest correlation [4]. Proliferation of granule cell precursors in the external granular layer (EGL) and their inward movement to form the internal granular layer (IGL) constitute a critical event in cerebellar development [12]. Sonic hedgehog (Shh), a mitotic factor secreted by Purkinje neurons, is responsible for the growth and patterning of the cerebellum [13]. IGF-1, an anabolic and neuroprotective factor, is essential for fetal growth and pre- and postnatal brain development [14]. IGF-1 has been suggested to have a supporting role in Shh-induced proliferation of EGL precursor cells [15].

We hypothesized that preterm birth and the subsequent loss of placental support with a resulting decrease

Table 1. Number of rabbit pups according to experimental group and postnatal age

	E29	P0	P2	P5	P9
Preterm pups	8	8	5	6	5
Term pups		10	10	6	6

E29, day of birth for preterm pups delivered by cesarean section; P0, day of birth for vaginally delivered term control pups and term equivalent age for preterm pups; P, postnatal day.

in circulating levels of IGF-1, may be essential, and partly causal, in the decreased cerebellar growth observed in very preterm infants.

Greater understanding of these relationships would be facilitated by an animal model that incorporates preterm birth. To date, few, if any, reported animal models incorporate the premature loss of placental support during a window of brain maturation corresponding to that of the very preterm human infant. The preterm rabbit pup model could be such an animal model since a significant part of the EGL proliferation takes place prior to birth, as opposed to mice and rats where EGL proliferation is mainly postnatal.

We thus aimed to evaluate if preterm delivery of rabbit pups would result in decreased circulating levels of IGF-1, and if preterm delivery per se would result in maturational changes in key elements of the developing cerebellum.

Material and Methods

The Preterm Rabbit Pup Model

The animal protocol was approved by the Swedish Animal Ethics Committee in Lund. The study included 64 rabbit pups from 16 litters. A half-breed between the New Zealand White and Lop was used. The preterm (PT) rabbit pups were delivered by cesarean section at gestational day 29 (term 32 days) after the does were anesthetized with i.v. propofol (5 mg/kg) and local infiltration of the abdominal wall using Lidocaine with adrenaline (10 mg/mL + 5 µL/mL). After birth, the pups were dried vigorously and placed and cared for in a closed infant incubator with humidified air: initially at 37°C and 70% humidity on day 1, 36°C and 60% humidity on day 2, and thereafter at 35°C and 50% humidity. At 2 h of age, the pups were weighed and hand-fed with 1 mL of kitten-milk replacement formula (KMR; PETAG Inc., USA) using a 3.5-Fr feeding tube, and every 12 h thereafter, the meals were increased by 0.5 mL. The term (T) pups were delivered at term by spontaneous vaginal delivery at gestational day 32, and then nursed and fed by their lactating doe. All pups were weighed once daily. The number of pups according to postnatal age in the PT and T groups is shown in Table 1. Mortality after inclusion in the study was 38% in the PT group and 0% in the T group.

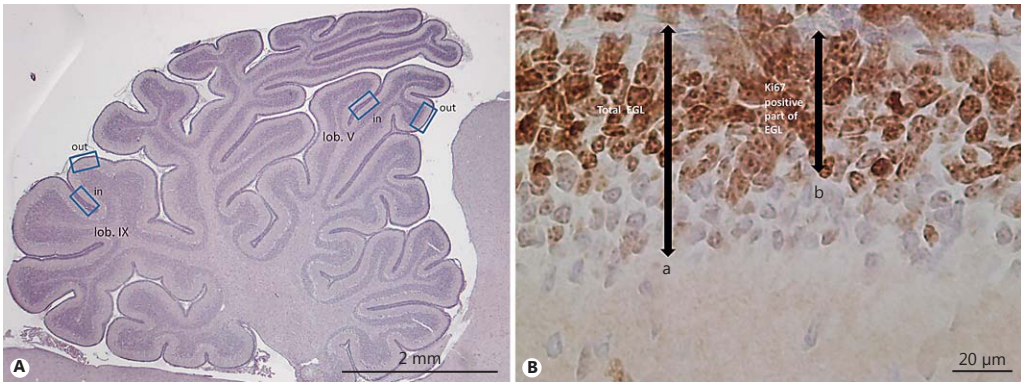


Fig. 1. Measurement of external granular layer (EGL) width. **A** Cerebellar overview illustrating areas (outward and inward regions of lobuli V and IX) used for quantitative measurement of total and proliferative EGL width. **B** HE- and Ki67-stained section of the EGL illustrating measurements obtained for quantitative

EGL analysis: (a) total EGL = width of proliferative EGL + inner zone of differentiated granular precursor cell (GPC) layer, and (b) proliferative EGL = width of Ki67-positive proliferating GPC layer.

Procedures for Blood and Tissue Sampling

Blood sampling was performed through cardiac puncture after sedation with isoflurane inhalation prior to *in vivo* perfusion fixation. Blood was collected in serum tubes and centrifuged at 1,000 g for 10 min at room temperature. The serum was then transferred into new tubes and stored at -80°C until analysis. Perfusion-fixation of the brain was performed by cardiac cannulation following thoracotomy and infusion of 0.9% saline followed by 4% paraformaldehyde (PFA). After complete perfusion, the cerebrum and cerebellum were carefully extracted from the skulls and immersed in 4% PFA. A change to fresh PFA was done after 3–6 h. Thereafter, the tissues were prepared for histochemical analysis as described below.

Serum IGF-1 Concentrations

The rabbit pup serum concentration of IGF-1 was determined using the human IGF-1 ELISA from Mediagnost (Reutlingen, Germany). The manufacturer has proved this assay applicable for rabbit serum samples. The analysis was performed according to the manufacturer's instructions.

Tissue Preparation

The brains were fixated in 4% PFA for 48 h. Thereafter, they were dehydrated, cleared, and infiltrated with paraffin automatically in a TISSUE-TEK V.I.P. (Miles Scientific Corp., Newark, NJ, USA) and embedded in paraffin. The cerebellum was sectioned, and 4-μm sections in the parasagittal plane at the level of the dentate nucleus were made (Leica, RM2255 Microtome) and mounted on microscope slides and dried at 37°C for 12–16 h.

Immunohistochemical Staining

Following deparaffinization, the sections were antigen-retrieval pretreated by boiling in 0.05 M boric acid buffer (pH 8.0) for 20

min followed by 20 min acclimatization at room temperature. Sections were then incubated with the primary antibody; mouse anti Ki67 (diluted 1:100, Dako, M7240), rabbit anti-cleaved caspase-3 (diluted 1:100, Cell signaling, #9661), mouse anti-calbindin (diluted 1:200, DBS, CB-855), rabbit anti-GFAP (diluted 1:150, Abcam, ab16997), mouse anti-Olig-2 (diluted 1:1,000, Millipore, MABN50), rabbit anti-Iba1 (diluted 1:200, Biocare, CP290), or rabbit anti-sonic hedgehog (diluted 1:200, Abcam, ab73958). All primary antibodies were diluted in PBS containing 5% normal goat serum (Jackson ImmunoResearch, 005-000-121) for 1 h at room temperature. Sections were then washed 3 times in PBS followed by incubation in corresponding secondary antibody, i.e. for rabbit primary antibodies: BrightVision rabbit/HRP (Immunologic, DPVR110HRP) and mouse primary antibodies: BrightVision mouse/HRP (Immunologic, DPVM110HRP), for 30 min. After being washed 3 times in Tris buffer (0.05 M, pH 7.6), the sections were incubated with DAB (diaminobenzidine, Sigma D5637-5G) for 5 min, counterstained with hematoxylin for 5 s, dehydrated and mounted in Pertex, and coverslipped. Mayer's hematoxylin and eosin (HE) staining was performed in a Leica ST4040 automatic stainer.

Microscope Analysis and Data Documentation

Microscope analysis was performed with a Leica light microscope (DMRX) equipped with a digital camera (MC120 HD, Leica).

Histological Analysis

Analysis of the EGL was performed in 4 predefined regions: the inner and outer portion of lobule V, and the inner and outer portion of lobule IX, respectively (Fig. 1). These regions were chosen as the regions with possible maturational differences in EGL proliferation and in subsequent width. Measurement of the width of the proliferative EGL, as constituted by Ki67-positive cells, was

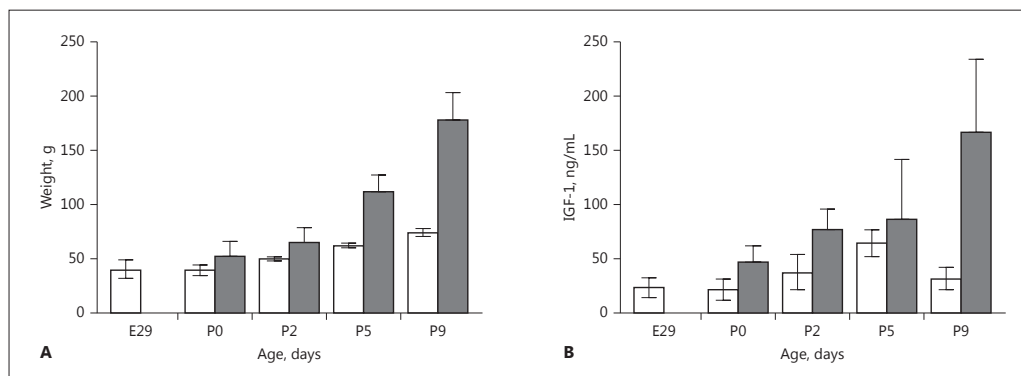


Fig. 2. Growth and serum insulin-like growth factor 1 (IGF-1) in preterm (PT) and term (T) rabbit pups. **A** Rabbit pups were weighed daily. The mean weight in the PT group (white bars) was lower than in the T group (grey bars) at all postnatal time points ($p < 0.05$). Error bars denote 1 SD. **B** Blood for analysis was retrieved at termination of PT and T pups at each respective postnatal

time point, and analyzed according to the description in Material and Methods. Mean levels of serum IGF-1 were lower in the PT group (white bars) than in the T group (grey bars) at all time points (all $p < 0.05$). Error bars denote 1 SD. Differences in weight and serum IGF-1 between groups were analyzed using the Mann-Whitney U test.

performed by using the $\times 40$ objective lens on the Leica DMRX microscope. The average of the 4 respective measured widths was calculated for each pup. Ki67-negative cells were regarded as differentiated, and were counted over an area of 100 μm . Qualitative evaluation was performed of Purkinje cell (calbindin immunoreactivity) and Bergmann glia morphology (GFAP immunoreactivity) at repeated postnatal ages in both the PT and T groups. Distribution and the presence of immunoreactivity for cleaved caspase-3 and Shh were described qualitatively.

Using the Leica Q500 image analysis system of the microscope, the area of calbindin-positive-stained cells was determined in relation to the area of the molecular layer. Thus, calbindin staining was expressed as percentage positive area in relation to a standardized area of the molecular layer. Nonspecific background staining was taken into consideration.

Cerebellar White-Matter Damage

Cerebellar white-matter impairment was evaluated in PT and T pups by HE staining. We further performed qualitative and quantitative analysis of immunostaining for Ki67, Olig2, and Iba1, for determination of cell proliferation and presence of oligodendroglial and microglial cells in PT and T pups.

Antibody Control for Immunohistochemical Staining

Antibody specificity tests were performed on parallel sections in all labeling experiments in which the primary antibodies were excluded from the labeling protocol (online suppl. Fig. 1; see www.karger.com/doi/10.1159/000480428 for all online suppl. material). This confirmed that the visualized and documented immunohistochemical staining was caused by the binding of the respective primary antibodies, not to the binding of secondary antibodies.

Statistics

Statistics were performed with IBM SPSS v21 for Microsoft Windows (IBM, Armonk, NY, USA). Differences between groups and time points were analyzed with the Mann-Whitney U test. Correlations between continuous variables were analyzed with regression analysis and adjustment for other variables was performed with multivariate regression analysis. $p < 0.05$ was considered significant.

Results

Weight Development

Preterm birth was associated with profound growth restriction (Fig. 2A). The mean weight of the PT group was less than that of the T group at each time point (all $p < 0.05$). The mean (\pm SD) weight of the PT group exhibited no increase in weight from birth at E29 to P0, i.e. 40 (± 3) versus 39 (± 2) g. Mean relative increase in weight from P0 to P9 was 3 times higher in the T group ($p < 0.01$; 246% in the T group vs. 83% in the PT group). No catch-up in weight in the PT group was noted during the observation period.

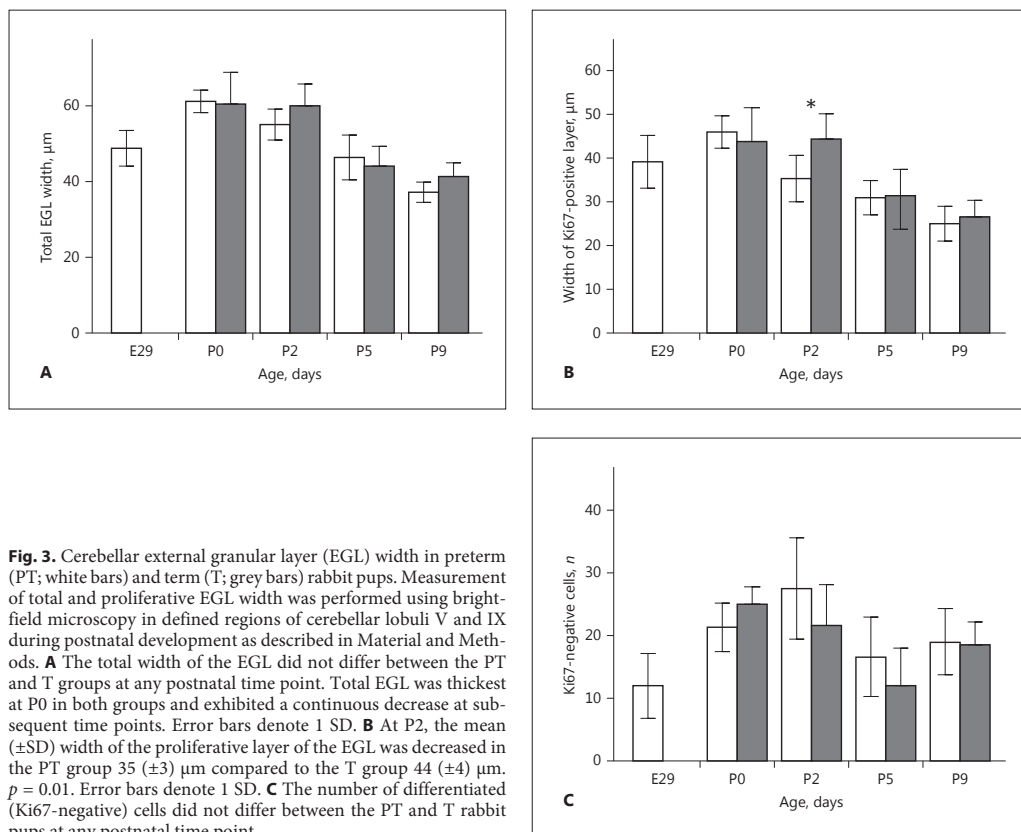


Fig. 3. Cerebellar external granular layer (EGL) width in preterm (PT; white bars) and term (T; grey bars) rabbit pups. Measurement of total and proliferative EGL width was performed using bright-field microscopy in defined regions of cerebellar lobuli V and IX during postnatal development as described in Material and Methods. **A** The total width of the EGL did not differ between the PT and T groups at any postnatal time point. Total EGL was thickest at P0 in both groups and exhibited a continuous decrease at subsequent time points. Error bars denote 1 SD. **B** At P2, the mean (\pm SD) width of the proliferative layer of the EGL was decreased in the PT group $35 (\pm 3) \mu\text{m}$ compared to the T group $44 (\pm 4) \mu\text{m}$. $p = 0.01$. Error bars denote 1 SD. **C** The number of differentiated (Ki67-negative) cells did not differ between the PT and T rabbit pups at any postnatal time point.

Serum IGF-1

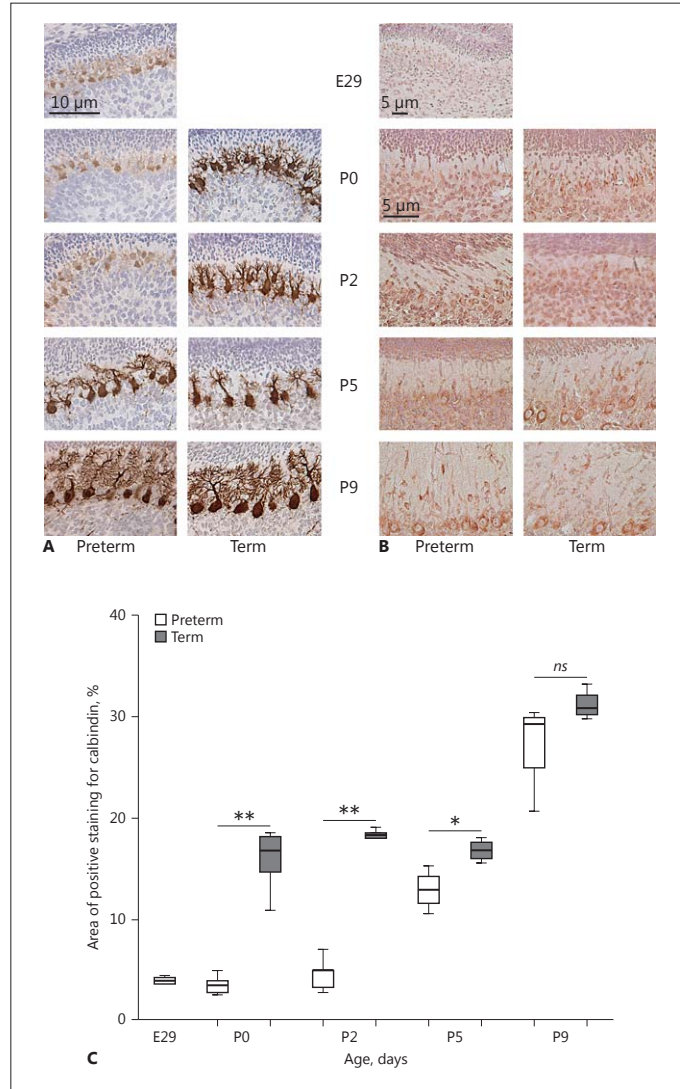
Mean protein levels of circulating IGF-1 were lower in the PT group than in the T group at P0, P2, and P9 (all $p < 0.05$; Fig. 2B). Mean serum values generally increased with increasing postnatal age in PT and in T pups, although a decrease was observed in mean serum IGF-1 level in the PT group at P9 compared to at P5 ($p < 0.05$). Concentrations of serum IGF-1 were highly correlated with weight ($r^2 = 0.89$, $p < 0.001$). This association remained significant ($p < 0.001$) after adjustment for gestational age at birth and for postnatal age.

Cerebellar Histology

EGL Measurements

There was no significant difference in the total width of the EGL between the PT and T groups. The total EGL width was largest at P0 in both groups and decreased at subsequent time points (Fig. 3A). The mean (\pm SD) Ki67-positive portion of the EGL was reduced at P2 in the PT group, i.e. $35 (\pm 3) \mu\text{m}$, versus in the T group, $44 (\pm 4) \mu\text{m}$ ($p = 0.01$; Fig. 3B). The mean Ki67-positive layer had its maximal width at P0 in the PT group and was decreased at P2 ($p < 0.05$). The corresponding decrease from maximal Ki67-positive width appeared later in the T group, between P2 and P5. The number of Ki67-negative cells in the EGL did not differ between the PT and T group at any of the observed time points (Fig. 3C).

Fig. 4. Cerebellar Purkinje cell development and Sonic hedgehog (Shh) expression in preterm (PT) and term (T) rabbit pups. **A** The development of Purkinje cells was evaluated by their morphology as determined by calbindin staining using a bright-field microscope as described in Material and Methods. Cell maturity over time, i.e. dendritic length and arborization, and their formation into the Purkinje cell layer were compared between the 2 groups at each postnatal time point. Representative immunosections of Purkinje cell morphology in PT and T pups are shown at each postnatal time point and also the progressive maturation from a fuciform to a stellate state. Calbindin staining (brown) was decreased in the PT group at P0 and P2 (vs. in the T group), with reduced arborization and dendritic spines. **B** Immunostaining for Shh was performed in sections from PT and T pups at each postnatal time point as described in Material and Methods. The pattern and intensity of staining for Shh were similar in the PT and T groups. Shh staining was scattered and present within the external granular layer, like in the internal granular layer, at early postnatal time points, and was restricted to the Purkinje cell somata (brown) at later time points. Scale bar, 50 μ m. **C** Quantitative analysis of calbindin staining showed a decrease in the area (%) of calbindin-labeled Purkinje cells in the PT pups versus in the T pups at P0 ($p = 0.003$), P2 ($p = 0.004$), and P5 ($p = 0.04$).

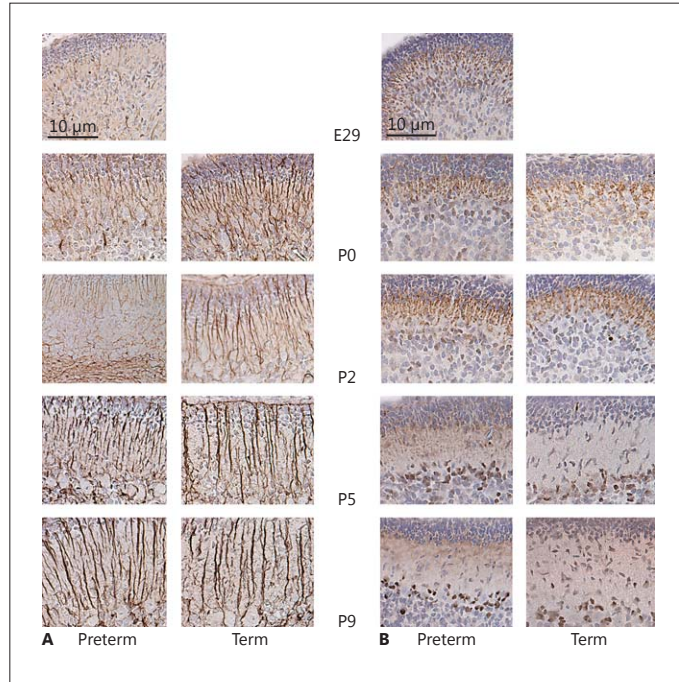


Purkinje Cell Maturation and Sonic Hedgehog Expression

Purkinje cell appearance, as determined by calbindin staining, changed strikingly over time, with an organized

monolayer of cell somata visible at P5, and still more defined at P9, in PT and T pups. Dendritic length and arborization increased with increasing postnatal age. Calbindin staining was less intense and Purkinje cell morphol-

Fig. 5. Cerebellar Bergman glia development and expression of cleaved caspase-3 in preterm (PT) and term (T) rabbit pups. **A** Immunostaining with GFAP was used to evaluate Bergman glia development in PT and T rabbit pups at each postnatal time point as described in Material and Methods. No clear differences in Bergman glia morphology were observed between the PT and T groups. At E29, GFAP-positive glial fibers were poorly defined and very scarce. With increasing postnatal age, glial fibers become more developed and confined to the molecular layer. **B** Staining for cleaved caspase-3 was applied to evaluate possible apoptosis during cerebellar development in the PT and T groups, as described in Material and Methods. Positive staining for cleaved caspase-3 (brown) was restricted to the radial fibers of the Bergman glia at E29, P0, and P2, and was predominantly located at the interface between the inner external granular layer and the molecular layer. At P5 and P9, staining for cleaved caspase-3 was restricted to the somata of cells, with a localization suggestive of Bergmann glia. The staining pattern for cleaved caspase-3 suggested a constitutive expression during Bergmann glia development and did not differ between the PT and T groups. Scale bar, 10 μ m.



ogy was clearly affected in the PT group at P0 and P2, with reduced arborization and dendritic spines compared to in the T group (Fig. 4). Quantitative analysis showed a clear decrease in percentage calbindin-labeled Purkinje cell area in the PT pups compared to in the T pups at P0 ($p = 0.003$), P2 ($p = 0.004$), and P5 ($p = 0.04$).

Staining for Shh revealed no clear difference between the PT and T groups. At earlier time points, it was more scattered and less pronounced in Purkinje cells and also visible within the EGL and the IGL. With increasing postnatal age, it was more intense and mainly confined to the cytoplasm of the Purkinje cell somata (at P5 and P9; Fig. 4).

Bergmann Glia

At E29, GFAP-positive glial fibers were poorly defined and very scarce. From P0 onwards, no obvious differences could be observed between the PT and T groups. With increasing postnatal age, the glial fibers were increasingly confined to the molecular layer (Fig. 5).

Cleaved Caspase-3

Staining for cleaved caspase-3 was positive in Bergmann glial fibers at E29, P0, and P2, and most pronounced at the interface between the inner EGL and the molecular layer. At later time points, i.e. P5 and P9, staining for cleaved caspase-3 was restricted to the somata of cells, with localization suggestive of Bergmann glia (Fig. 5).

Staining for cleaved caspase-3 was not observed in the EGL or in other cellular populations, apart from those with a Bergmann glia appearance. No difference in cleaved caspase-3 staining was observed between the PT and T pups.

Cerebellar White-Matter Impairment

At P2 and P5, several of the PT pups exhibited signs of damage localized to the cerebellar white matter, which was not observed in the T group (Fig. 6). Quantitative densitometric analysis in PT and T pups at P2 showed a reduction in Olig2-positive cells in cerebellar white mat-

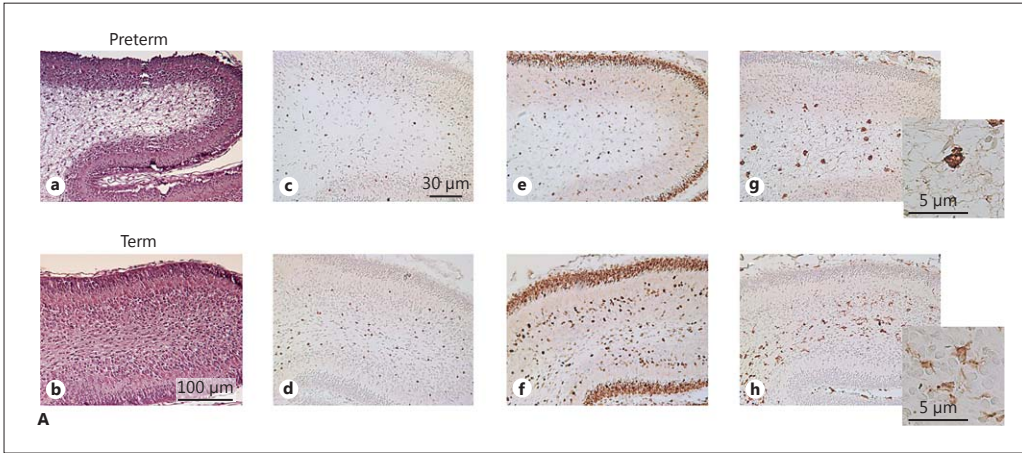
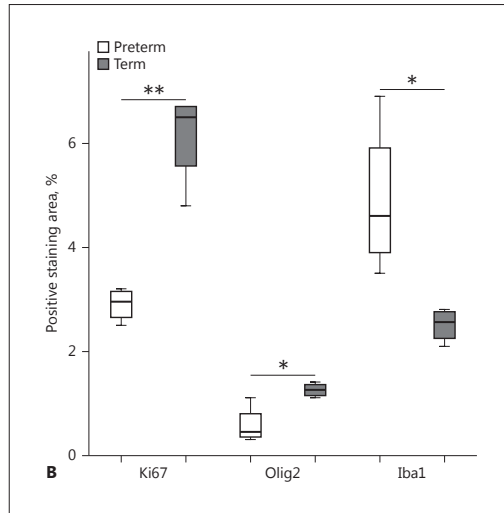


Fig. 6. Cerebellar white-matter damage in preterm (PT) rabbit pups. **A** Cerebellar sections exhibited signs of cellular damage in cerebellar white matter in PT pups at P2 and P5. HE. Cerebellar white-matter damage was not observed in T pups. Immunostaining with antibodies against Olig2, Ki-67, and Iba1, respectively, were performed as described in Material and Methods to characterize proliferation and oligodendroglial and microglial cellular response in cerebellar white matter in PT and T rabbit pups. Upper row: HE (**a**), Olig2 (**c**), Ki67 (**e**) and Iba1 (**g**) staining, respectively, in a representative PT rabbit pup at P2. Signs of white-matter damage in the HE-stained section correspond to a marked reduction of proliferating cells, a reduced number of Olig2-positive cells, and an increased number of Iba1-positive microglia in the cerebellar white matter. Lower row: the corresponding immunostainings in a T pup at P2 (**b**, **d**, **f**, **h**) with no signs of white-matter damage for comparison. Scale bars: 100 μ m (HE); 30 μ m (Ki67, Olig2, and Iba1). **Insets** An activated ameboid Iba1-positive microglial morphology in the PT pup (**g**) compared to a quiescent ramified morphology in the T pup (**h**). **B** Quantitative analysis showed a decrease in staining for Olig2 (** $p = 0.009$) and Ki67 (* $p = 0.03$) and an increase in staining for Iba1 (* $p = 0.04$).



ter ($p = 0.009$) and a decrease in proliferating Ki67-positive cells ($p = 0.03$). Immunostaining with Iba1 revealed an increased number of Iba1-positive microglia ($p = 0.04$) in cerebellar white matter, with an activated ameboid morphology compared to ramified, quiescent cells in the T pups (Fig. 6).

Discussion

We have shown that preterm birth in the rabbit pup mimics birth in the very preterm human infant in several relevant aspects. Cesarean section of the pregnant doe, 3 days prior to vaginal delivery, was associated with postnatal growth restriction and with decreased postnatal circulating levels of IGF-1 in preterm rabbit pups. These

findings were accompanied by changes in EGL proliferation and cerebellar Purkinje cell morphology, indicating affected or impaired cerebellar development. Of note, we also observed signs of cerebellar white-matter damage in a subgroup of preterm rabbit pups.

The vast majority of studies aiming to evaluate the consequences of insults in the very immature brain have used mice or rat models. These rodents, when studied at postnatal age P2–P7, exhibit a varying degree of brain immaturity reflecting that of the human preterm brain from extreme prematurity to a maturity corresponding to term age [16]. However, there is strong support that the harmful influence of very preterm birth on brain development is related to systemic alterations due to premature loss of the maternal/fetal interaction, e.g., placental support and trophic deprivation due to less than optimal postnatal nutrition [17]. An animal model that incorporates both brain immaturity per se and trophic deprivation due to loss of placental support would thus seem essential in order to generate knowledge which can be translated to the preterm human infant. The preterm rabbit pup model has primarily been used by us and other study groups for the study of cerebral intraventricular hemorrhage [10, 18]. Unlike other rodent models, it incorporates premature loss of placental support, so we aimed to evaluate aspects of trophic deprivation, i.e. circulating levels of IGF-1 and postnatal weight development.

Preterm birth in rabbit pups was followed by decreased circulating levels of IGF-1 and by postnatal growth restriction. Circulating levels of IGF-1 were highly correlated to body weight independently of postnatal age and maturity at birth. These results correspond well to what we and other authors have shown in preterm human infants [17]. Very preterm infants have low levels of IGF-1 after birth and generally exhibit an increase at around 30 weeks' gestation which coincides with the initiation of catch-up growth. Variations in protein and caloric intake have a limited influence on circulating IGF-1 during the initial phase of growth restriction but a significant influence on the IGF-1 levels during established catch-up growth. The rabbit pups in this study received a nutritional intake relative to weight very similar to that administered to human infants. The group of term pups that served as a reference group for normal growth received milk from their lactating doe. The study was not designed to evaluate the impact of varied nutrition on either IGF-1 or on body growth. Although body weight increased with increasing postnatal age in PT pups, there was no sign of accelerated catch-up growth. The observed decrease in serum IGF-1 from P5

to P9 was most likely representative of a relative nutritional deficit.

IGF-1, by acting through the IGF-1 receptor, influences all of the mechanisms in normal brain development apart from migration, i.e. proliferation, differentiation, maturation, and apoptosis [19–21]. The growth factor IGF-1 is predominantly expressed in neurons and is enrolled in progenitor proliferation and neuronal outgrowth in the cerebellum [22]. IGF-1 has been shown to act on the survival of the Purkinje cells via an antiapoptotic effect [22] and also on the proliferation of the granule cell precursors [23]. The decrease in circulating levels of IGF-1 following the loss of placental support at preterm birth in human infants is associated with lower brain volumes at term age with a positive correlation to cerebellar volume [4].

The cerebellum is the fastest-growing part of the brain during the last trimester of human gestation and is in a very active proliferation phase before reaching term age which suggest a heightened vulnerability to environmental changes [8, 12]. Indeed, we have shown that cerebellar growth at term age, as detected by volumetric magnetic resonance imaging, exhibits a stronger association with postnatal growth and longitudinal levels of circulating IGF-1 than that of the supratentorial brain in very preterm infants [4]. Effects of trophic deprivation in the immature cerebellum have primarily been studied in animal models of intrauterine growth restriction (IUGR). IUGR induced by uterine artery ligation in the guinea-pig leads to a significant reduction in the number of Purkinje cells and the volume of the molecular layer, the internal granular layer, and the cerebellar white matter [24].

In humans, placental insufficiency has been shown to alter brain development following IUGR [25] and to reduce the number of Purkinje cells in the cerebellum [24].

The longitudinal evaluation of total and proliferative EGL width in rabbit pups showed that total EGL width was maximal at P0 in both groups and decreased thereafter. Measure of the proliferative portion of the EGL, as determined by Ki67-positive staining, indicated that proliferation was maximal around P0–P2. The finding that the PT group exhibited a decreased proliferative layer at P2 was not unexpected, as the period of maximal proliferation during development probably represents a time point of heightened vulnerability. Our observations of the EGL of rabbit pups are similar to those described in human cerebellar maturation; the EGL was thickest at P0, which is analogous to the developmental stage of the human fetal brain at 25 weeks' gestation when it reaches its peak thickness and is highly proliferative [12].

We observed altered Purkinje cell morphology clearly in the PT group, with decreased calbindin staining, reduced cell somata size, and decreased arborization. These morphological changes were most prominent at P0 and P2, coinciding in time with the finding of decreased EGL proliferation. The vulnerability of Purkinje cell development to insults associated with preterm birth have been recently shown in rat pups exposed to neonatal hyperoxia and also in preterm rabbit pups with preterm cerebral intraventricular hemorrhage exhibiting delayed maturation of Purkinje cells and decreased granular precursor cell proliferation [26, 27]. The cerebellar Purkinje cells have several key functions relating to EGL proliferation, and subsequent inward cell migration and Purkinje cell secretion of the transcription factor Shh are key for EGL precursor cell proliferation [12, 13, 28]. We did not detect any clear differences in Shh staining between the PT and T groups, and thus do not have evidence of Purkinje cell degeneration leading to impaired or reduced Shh-induced proliferation. However, our evaluation was restricted to the ligand Shh whereas alterations in other key players, such as the Patched receptor on granule precursor cells or intracellular mediators of transcription, may be involved in decreased granule cell proliferation.

We observed that Shh was diffusely present in the EGL and inner layers at earlier time points, and that with increasing postnatal age it became increasingly confined to an organized monolayer of Purkinje cell somata. This maturational pattern has previously been observed in mice and in human embryos [16, 28].

We hypothesized that events relating to premature exposure to the postnatal environment would induce mechanisms leading to apoptosis in the rapidly proliferating cerebellum. This was not observed, as determined by staining for cleaved caspase-3. However, we did detect positive staining for cleaved caspase-3 which was restricted to the Bergmann glia. Previous studies have demonstrated that active caspase-3 has a role of inducing and maintaining differentiation of the Bergmann glia during normal cerebellar development [29–31]. The distribution and staining pattern over time in Bergmann glia did not differ between PT and T pups.

Finally, we observed clear signs of damage in the cerebellar white matter in PT pups. This damage was characterized by a reduction in Olig2-staining oligodendrocytes, a reduced number of proliferating Ki67-positive cells, and an increased presence of Iba1-positive microglia with an activated morphology. These findings are very similar to those observed in immature, periventricular,

supratentorial white matter following exposure to proinflammatory or hyperoxic insults [32, 33]. Less is known about the possible intrinsic vulnerability of the cerebellar white-matter preoligodendrocyte population. Of note, selective cerebellar white-matter volume reduction has been observed in adolescents with a history of very preterm birth and prenatal growth restriction (pers. communication).

In conclusion, we show that the preterm rabbit pup model exhibits a combination of characteristics relevant to human preterm birth. Our study is descriptive and causality can thus not be inferred between our observations of decreased growth, circulating IGF-1, and alterations in key cerebellar cell populations suggestive of impaired cerebellar development. Future studies on this animal model will focus on relating our observations to long-term functional evaluation. We also propose the preterm rabbit pup model as appropriate for the study of relationships between nutritional modification, pharmacological intervention, and short- and long-term neurodevelopment.

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References

- 1 Wood NS, Marlow N, Costeloe K, Gibson AT, Wilkinson AR; EPICure Study Group: Neurologic and developmental disability after extremely preterm birth. *N Engl J Med* 2000; 343:378–384.
- 2 Marret S, Marchand-Martin I, Picaud JC, Hascoët JM, Arnaud C, Rozé JC, Truffert P, Larroque B, Kaminski M, Ancel PY; EPIPAGE Study Group: Brain injury in very preterm children and neurosensory and cognitive disabilities during childhood: the EPIPAGE cohort study. *PLoS One* 2013;8:e62683.
- 3 Serenius F, Källen K, Blennow M, Ewald U, Fellman V, Holmström G, Lindberg E, Lundqvist P, Marsál K, Norman M, Olhager E, Stigson L, Sjöqvist K, Vollmer B, Strömberg B; EXPRESS Group: Neurodevelopmental outcome in extremely preterm infants at 2.5 years after active perinatal care in Sweden. *JAMA* 2013;309:1810–1820.

- 4 Hansen-Pupp I, Hövel H, Hellström A, Hellström-Westas L, Löfqvist C, Larsson EM, Lazeyras F, Fellman V, Hüppi PS, Ley D: Postnatal decrease in circulating insulin-like growth factor-I and low brain volumes in very preterm infants. *J Clin Endocrinol Metab* 2011;96:1129–1135.
- 5 Messerschmidt A, Brugger PC, Boltshauser E, Zoder G, Sterniste W, Birnbacher R, Prayer D: Disruption of cerebellar development: potential complication of extreme prematurity. *Am J Neuroradiol* 2005;26:1659–1667.
- 6 Tam EW, Ferriero DM, Xu D, Berman JJ, Vigneron DB, Barkovich AJ, Miller SP: Cerebellar development in the preterm neonate: effect of supratentorial brain injury. *Pediatr Res* 2009;66:102–106.
- 7 Tam EW, Rosenbluth G, Rogers EE, Ferriero DM, Glidden D, Goldstein RB, Glass HC, Piecuch RE, Barkovich AJ: Cerebellar hemorrhage on magnetic resonance imaging in preterm newborns associated with abnormal neurologic outcome. *J Pediatr* 2011;158:245–250.
- 8 Limperopoulos C, Soul JS, Gauvreau K, Huppi PS, Warfield SK, Bassan H, Robertson RL, Volpe JJ, du Plessis AJ: Late gestation cerebellar growth is rapid and impeded by premature birth. *Pediatrics* 2005;115:688–695.
- 9 Vinukonda G, Csiszar A, Hu F, Dummula K, Pandey NK, Zia MT, Ferreri NR, Ungvari Z, LaGamma EF, Ballabh P: Neuroprotection in a rabbit model of intraventricular haemorrhage by cyclooxygenase-2, prostanoïd receptor-1 or tumour necrosis factor- α inhibition. *Brain* 2010;133:2264–2280.
- 10 Chua CO, Chahboune H, Braun A, Dummula K, Chua CE, Yu J, Ungvari Z, Sherbany AA, Hyder F, Ballabh P: Consequences of Intraventricular hemorrhage in a rabbit pup model. *Stroke* 2009;40:3369–3377.
- 11 Hansen-Pupp I, Hövel H, Löfqvist C, Hellström-Westas L, Cilio CM, Andersson S, Fellman V, Ley D: Circulatory insulin-like growth factor-I and brain volumes in relation to neurodevelopmental outcome in very preterm infants. *Pediatr Res* 2013;74:564–569.
- 12 Volpe JJ: Cerebellum of the premature infant: rapidly developing, vulnerable, clinically important. *J Child Neurol* 2009;24:1085–1104.
- 13 Dahmane N, Ruiz i Altaba A: Sonic hedgehog regulates the growth and patterning of the cerebellum. *Development* 1999;126:3089–3100.
- 14 Langford K, Nicolaides K, Miell JP: Maternal and fetal insulin-like growth factors and their binding proteins in the second and third trimesters of human pregnancy. *Hum Reprod* 1998;13:1389–1393.
- 15 Fernandez C, Tatar VM, Bertrand N, Dahmane N: Differential modulation of sonic hedgehog-induced cerebellar granule cell precursor proliferation by the IGF signaling network. *Dev Neurosci* 2010;32:59–70.
- 16 Haldipur P, Bharti U, Govindan S, Sarkar C, Iyengar S, Gressens P, Mani S: Expression of sonic hedgehog during cell proliferation in the human cerebellum. *Stem Cells Dev* 2012;21:1059–1068.
- 17 Hansen-Pupp I, Löfqvist C, Polberger S, Niklasson A, Fellman V, Hellström A, Ley D: Influence of insulin-like growth factor I and nutrition during phases of postnatal growth in very preterm infants. *Pediatric Research* 2011;69:448–453.
- 18 Sveinsdóttir S, Cinthio M, Ley D: High-frequency ultrasound in the evaluation of cerebral intraventricular haemorrhage in preterm rabbit pups. *Ultrasound Med Biol* 2012;38:423–431.
- 19 Carson MJ, Behringer RR, Brinster RL, McMorris FA: Insulin-like growth factor I increases brain growth and central nervous system myelination in transgenic mice. *Neuron* 1993;10:729–740.
- 20 Chrysis D, Calikoglu AS, Ye P, D'Ercole AJ: Insulin-like growth factor-I overexpression attenuates cerebellar apoptosis by altering the expression of Bcl family proteins in a developmentally specific manner. *J Neurosci* 2001;21:1481–1489.
- 21 O'Kusky JR, Ye P, D'Ercole AJ: Insulin-like growth factor-I promotes neurogenesis and synaptogenesis in the hippocampal dentate gyrus during postnatal development. *J Neurosci* 2000;20:8435–8442.
- 22 Croci L, Barili V, Chia D, Massimino L, van Vugt R, Masserdotti G, Longhi R, Rotwein P, Consalez GG: Local insulin-like growth factor I expression is essential for Purkinje neuron survival at birth. *Cell Death Differ* 2011;18:48–59.
- 23 Ye P, Xing Y, Dai Z, D'Ercole AJ: In vivo actions of insulin-like growth factor-I (IGF-I) on cerebellum development in transgenic mice: evidence that IGF-I increases proliferation of granule cell progenitors. *Brain Res Dev Brain Res* 1996;95:44–54.
- 24 Mallard C, Loeliger M, Copolov D, Rees S: Reduced number of neurons in the hippocampus and the cerebellum in the postnatal guinea-pig following intrauterine growth restriction. *Neuroscience* 2000;100:327–333.
- 25 Tolsa CB, Zimine S, Warfield SK, Freschi M, Sancho Rossignol A, Lazeyras F, Hanquinet S, Pfizenmaier M, Hüppi PS: Early alteration of structural and functional brain development in premature infants born with intrauterine growth restriction. *Pediatr Res* 2004;56:132–138.
- 26 Scheuer T, Sharkovska Y, Tarabykin V, Marggraf K, Brockmüller V, Bühner C, Endesfelder S, Schmitz T: Neonatal hyperoxia perturbs neuronal development in the cerebellum. *Mol Neurobiol* 2017, E-pub ahead of print.
- 27 Agyemang AA, Sveinsdóttir K, Vallius S, Sveinsdóttir S, Bruschetini M, Romantsik O, Hellström A, Smith LEH, Ohlsson L, Holmqvist B, Gram M, Ley D: Cerebellar exposure to cell-free hemoglobin following preterm intraventricular hemorrhage: causal in cerebellar damage? *Transl Stroke Res* 2017, Epub ahead of print.
- 28 Lewis PM, Gritti-Linde A, Smeys R, Kottmann A, McMahon AP: Sonic hedgehog signaling is required for expansion of granule neuron precursors and patterning of the mouse cerebellum. *Dev Biol* 2004;270:393–410.
- 29 Oomman S, Strahlendorf H, Finckbone V, Strahlendorf J: Non-lethal active caspase-3 expression in Bergmann glia of postnatal rat cerebellum. *Brain Res Dev Brain Res* 2005;160:130–145.
- 30 Oomman S, Strahlendorf H, Dertien J, Strahlendorf J: Bergmann glia utilize active caspase-3 for differentiation. *Brain Res* 2006;1078:19–34.
- 31 Finckbone V, Oomman SK, Strahlendorf HK, Strahlendorf JC: Regional differences in the temporal expression of non-apoptotic caspase-3-positive Bergmann glial cells in the developing rat cerebellum. *Front Neuroanat* 2009;3:1–7.
- 32 Gerstner B, DeSilva TM, Genz K, Armstrong A, Brehmer F, Neve RL, Felderhoff-Mueser U, Volpe JJ, Rosenberg PA: Hyperoxia causes maturation-dependent cell death in the developing white matter. *J Neurosci* 2008;28:1236–1245.
- 33 Supramaniam V, Vontell R, Srinivasan L, Wyatt-Ashmead J, Hagberg H, Rutherford M: Microglia activation in the extremely preterm human brain. *Pediatr Res* 2013;73:301–309.

Paper II



Cerebellar Exposure to Cell-Free Hemoglobin Following Preterm Intraventricular Hemorrhage: Causal in Cerebellar Damage?

Alex Adusei Agyemang¹ · Kristbjörg Sveinsdóttir¹ · Suvi Vallius¹ ·
Snjolaug Sveinsdóttir¹ · Matteo Bruschetti¹ · Olga Romantsik¹ · Ann Hellström² ·
Lois E. H. Smith³ · Lennart Ohlsson⁴ · Bo Holmqvist⁵ · Magnus Gram^{1,6} · David Ley¹

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Abstract Decreased cerebellar volume is associated with intraventricular hemorrhage (IVH) in very preterm infants and may be a principal component in neurodevelopmental impairment. Cerebellar deposition of blood products from the sub-arachnoid space has been suggested as a causal mechanism in cerebellar underdevelopment following IVH. Using the pre-term rabbit pup IVH model, we evaluated the effects of IVH induced at E29 (3 days prior to term) on cerebellar development at term-equivalent postnatal day 0 (P0), term-equivalent postnatal day 2 (P2), and term-equivalent postnatal day 5 (P5). Furthermore, the presence of cell-free hemoglobin (Hb) in cerebellar tissue was characterized, and cell-free Hb was evaluated as a causal factor in the development of cerebellar damage following preterm IVH. IVH was associated with a decreased proliferative (Ki67-positive) portion of the external granular layer (EGL), delayed Purkinje cell maturation, and

activated microglia in the cerebellar white matter. In pups with IVH, immunolabeling of the cerebellum at P0 demonstrated a widespread presence of cell-free Hb, primarily distributed in the white matter and the molecular layer. Intraventricular injection of the Hb scavenger haptoglobin (Hp) resulted in a corresponding distribution of immunolabeled Hp in the cerebellum and a partial reversal of the damaging effects observed following IVH. The results suggest that cell-free Hb is causally involved in cerebellar damage following IVH and that blocking cell-free Hb may have protective effects.

Keywords Intraventricular hemorrhage · Hemoglobin · Haptoglobin · Cerebellum · External granular layer

Introduction

Cerebral intraventricular hemorrhage (IVH) continues to be a serious complication of preterm birth, resulting in a high incidence of neurodevelopmental impairment, including cerebral palsy and intellectual disability [1]. During the past decades, neurological impairment following very preterm birth has primarily been considered to originate in cerebral white matter lesions [2, 3], but recent findings have also linked neurological deficits of preterm birth to cerebellar abnormalities [4, 5]. Prevalence of cerebellar injury has been described to be as high as 58% in infants with cerebral palsy following IVH and preterm birth [6].

From gestational weeks 20 to 40, the cerebellum undergoes an unparalleled growth with a volumetric increase from approximately 1 to 25 cm³ [7]. This rapid growth renders the cerebellum very sensitive to injury [8, 9]. Cerebellar underdevelopment may ensue from a direct cerebellar injury, such as hemorrhage or infarction, or from a secondary effect related to damage at a remote but connected area of the brain [10].

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✉ David Ley
david.ley@med.lu.se

¹ Pediatrics, Department of Clinical Sciences Lund, Skåne University Hospital, Lund University, BMC C14, SE-221 84 Lund, Sweden

² Department of Ophthalmology, Institute of Neuroscience and Physiology, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden

³ Department of Ophthalmology, Boston Children's Hospital, Harvard Medical School, Boston, MA, USA

⁴ MicroMorph Histology Services, Lund, Sweden

⁵ ImaGene-iT AB, Medicon Village, Lund, Sweden

⁶ Infection Medicine, Department of Clinical Sciences Lund, Lund University, Lund, Sweden

Cerebellar hypoplasia has repeatedly been shown to be associated with supratentorial IVH in very preterm infants and is a potential component in neurological disability [9, 11, 12]. Of note, the severity of IVH is linked to the degree of impaired cerebellar development in preterm infants, with cerebellar volume at term age being inversely correlated with increasing severity of IVH [7].

In clinical studies, MRI at term age shows infratentorial hemosiderin deposits in 70% of preterm infants with IVH and disrupted cerebellar development. The deposits are prominent not only on the cerebellar surface but also on the surface of the brain stem and in the region of the fourth ventricle. This hemosiderin deposition is the most predictive factor for impairment in cerebellar development and thus is suggested as a plausible causal mechanism of cerebellar hypoplasia following preterm IVH [9].

The neurotoxicity of cell-free hemoglobin (Hb) and its metabolites has been reported after intraventricular, intraparenchymal, and subarachnoid hemorrhage (SAH) [13–20]. Cell-free Hb and its metabolites free heme, iron, reactive oxygen species (ROS), and free radicals can be highly damaging to cells, lipids, proteins, and DNA through oxidative modification, fragmentation, and cross-linking [21–23]. Cell-free Hb and its metabolites can induce cytotoxic, oxidative, and inflammatory pathways in the cerebrospinal fluid (CSF) and choroid plexus ependyma leading to tissue damage and cell death following preterm rabbit pup IVH [17–19]. Furthermore, a high accumulation of cell-free Hb in the periventricular white matter has been observed following hemorrhage in the rabbit pup IVH model [20].

In this study, we have completed the first investigation of the exposure of the developing cerebellum to cell-free Hb following preterm IVH and the potentially damaging effect on cerebellar development. Furthermore, we report on the protective effects of the Hb scavenger haptoglobin (Hp) following intraventricular administration. Results show that after IVH, key cell populations of the developing cerebellum are exposed to cell-free Hb, which may be central in the pathophysiological events leading to cerebellar underdevelopment.

Materials and Methods

Animals

The study was approved by the Swedish Animal Ethics Committee in Lund. We used the well-established preterm rabbit pup model of glycerol-induced IVH as previously described [24]. The study included 59 rabbit pups from 9 litters delivered at gestational day 29 (full term corresponding to 32 days) [25, 26]. A half-breed between New Zealand White and Lop was used. The pups were delivered by caesarean section after the does were anesthetized with i.v. propofol

(5 mg/kg) and with local infiltration of the abdominal wall using lidocaine with adrenaline (10 mg/ml + 5 µl/ml, 20–30 ml). After delivery, the pups were dried, weighed, and placed in an infant incubator set to a temperature of 34–35 °C and ambient humidity. At 2 h of age, the pups were hand-fed with 2 ml (100 ml/kg/day) of kitten milk formula (KMR; PetAg Inc., Hampshire, IL, USA) using a 3.5 French feeding tube and fed every 12 h increasing each meal by 1 ml. At 2 h of age, the pups were injected intraperitoneally with 50% (v/v) sterile glycerol (6.5 g/kg; Teknova, Hollister, CA, USA) to induce IVH. Ultrasound imaging of the brain was performed at 6 h of age to grade the severity of the IVH and detect SAH and daily thereafter using the VisualSonics Vevo 2100 (VisualSonics Inc., ON, Canada) with a MS-550D 40 MHz transducer. Animals with IVH at 6 h were included in the IVH group, and those without detectable IVH at all time points were used as controls (denoted as sham control). The reproducibility and accuracy of high-frequency ultrasound in this animal model have been described previously [24].

Intraventricular Injections

After the initial ultrasound examination at 6 h of age, pups with IVH (presence of blood within distended lateral ventricles and no sign of parenchymal involvement) were randomized into one of the following three groups: IVH, IVH + Hp, or IVH + Vehicle. Pups in the IVH + Hp and IVH + Vehicle groups received an ultrasound-guided intraventricular injection at 8 h of age of either 20 µl of human Hp (50 mg/ml, Bio Products Laboratory, London, UK) or 20 µl of vehicle solution (9 mg/ml NaCl, Fresenius Kabi, Lake Zurich, IL, USA), using 27 G Hamilton syringes (Hamilton Robotics, Reno, NV, USA). The efficacy and accuracy of this method have been described previously [26]. The animals were euthanized at the following time points: 72 h (P0, corresponding to term-equivalent postnatal day 0), 120 h (P2, corresponding to term-equivalent postnatal day 2), or 192 h (P5, corresponding to term-equivalent postnatal day 5) of age. Cerebellar tissues were sampled and processed as described below. An overview of the study design is given in Fig. 1.

Tissue Collection and Processing

Following sedation with isoflurane inhalation, perfusion fixation of the brain was performed at P0 and P5 by cardiac cannulation following thoracotomy and infusion of 0.9% saline followed by 4% paraformaldehyde (PFA, buffered with phosphate buffer saline (PBS) 0.1 M, pH 7.4). After completed perfusion, the cerebrum and cerebellum were carefully extracted from the skulls and immersed in 4% PFA for a total of 48 h. SAH was confirmed in all pups with IVH with visible presence of hemorrhagic CSF covering the cerebellar cortex. None of the control pups exhibited macroscopic signs of

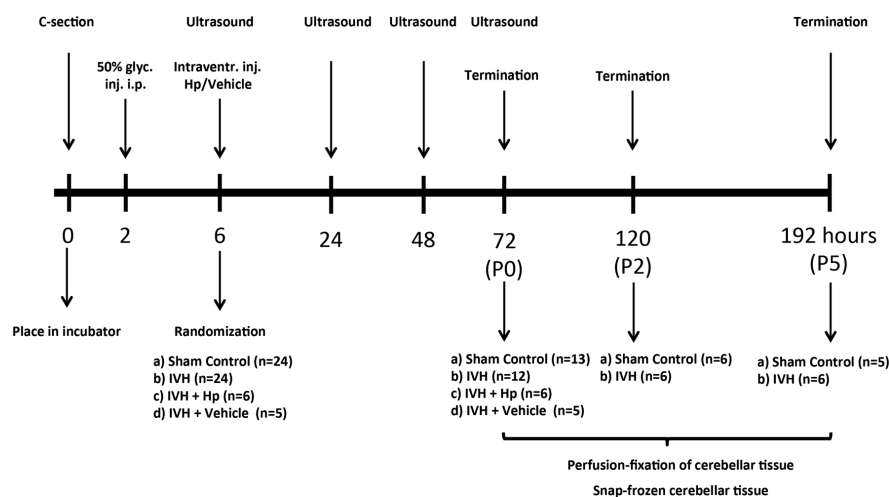


Fig. 1 Study outline. A diagram summarizing the experimental procedure. The experiment consisted of the following steps: preterm delivery of rabbit pups by caesarean section, induction of IVH by intraperitoneal glycerol administration, verification of IVH or sham

control by the use of high-frequency ultrasound, randomization into study groups, intraventricular administration of Hp or vehicle solution, termination of pups, and collection of cerebellar tissue. For details about each step, see “Materials and Methods”

SAH. A change to fresh PFA was performed after 3–6 h. Thereafter, the tissues were dehydrated, cleared, infiltrated with paraffin, and embedded in paraffin blocks. The cerebellum was sectioned into 4- μ m sections (Leica, RM2255 Microtome) in the parasagittal plane at the level of the dentate nucleus and mounted on microscope slides and dried at 37 °C for 12–16 h. None of the cerebellar samples in pups with IVH or in control pups exhibited signs of primary cerebellar hemorrhage. Prior to antibody staining for immunohistochemistry (IHC), the sections were rehydrated, followed by heat-induced antigen retrieval at 90–95 °C for 20 min either in boric acid buffer (pH 8.0) for labeling of Ki67, calbindin, and Iba1 or in citric acid (pH 6.0, with 0.05% Tween 20 or 0.2% Triton X) for 10–20 min for immunofluorescence labeling of Hb and Hp.

Immunofluorescence Labeling

Immunofluorescence labeling of Hb was performed to investigate the presence and distribution of both encapsulated erythrocytes and cell-free Hb within the cerebellum. Double immunofluorescence labeling of Hb together with human Hp was performed to simultaneously visualize Hb and Hp to elucidate whether the intraventricularly injected human Hp could reach the cerebellar brain regions containing Hb (preferentially the cell-free Hb) in the IVH rabbit pups.

In brief, the immunofluorescence labeling protocol was carried out as described below. Following antigen retrieval, sections were immersed in PBS (2 \times 5 min),

encircled with silicon (PAP-pen, Sakura, Tokyo, Japan), and then blocked with 1% bovine serum albumin (BSA) in PBS containing 0.05% Triton X (PBST \times BSA) for 60 min at room temperature (RT). This step was followed by 16 h of incubation at 4 °C with either one of the primary antibodies or a mixture of the two primary antibodies diluted in PBST \times BSA. All antibody incubations were performed in a moisture chamber. Primary antibodies used were against Hb, made in goat (diluted 1:500), and against human Hp, made in chicken (diluted 1:1000), both from GenWay Biotech, Inc. (San Diego, CA, USA) and diluted in PBST \times BSA. Sections were then rinsed in PBS (3 \times 3 min), followed by incubation for 60 min at RT with one secondary antibody made against goat IgG or with a mixture of secondary antibodies made against goat IgG and chicken IgY (diluted 1:200 in PBST \times BSA). The secondary antibodies were both affinity-purified Fab2 fragments for multi-labeling, made in donkey (Jackson ImmunoResearch, West Grove, PA, USA). The anti-chicken IgY was conjugated with Alexa Fluor 488 (AF488) and the goat IgG conjugated with Rhodamine Red (rhodamine). Sections were then rinsed in PBS (3 \times 3 min) and incubated in DAPI (0.1 μ M, diluted in PBS, Invitrogen, Rockford, IL, USA) for 30 min at RT. After being rinsed in PBS (3 \times 3 min), sections were mounted (Fluoroshield, Abcam, England, ab104135) and cover-slipped. All animal groups were always processed together in the same immunolabeling experiment.

Antibody Control for Immunofluorescence Labeling

Antibody specificity tests were performed on parallel sections in all labeling experiments; in these tests, the primary antibodies were excluded from the labeling protocol (Fig. 1 in the Data supplement). This control confirmed that the visualized and documented Hb and Hp immunofluorescence labeling (Fig. 2) was caused by binding of the respective primary antibodies and was not the result of binding of secondary antibodies or autofluorescence. All tested samples showed no Hb or Hp labeling within the cerebellum (see Fig. 1 in the Data supplement). Autofluorescence was solely obtained from whole cell bodies, from erythrocytes/RBCs in the subarachnoid space preferentially, and occasionally from some neuronal cell bodies. Thus, the antibody controls showed that both primary and secondary antibodies bind to their targets in the immunofluorescence labeling protocol applied here, supporting their specific detection of rabbit Hb and human Hp, visualized as extracellular (cell-free) and in whole erythrocytes.

Analyses of the Distribution of Hb and Its Relation to Hp

The anatomical distribution of the double immunofluorescence-labeled Hb and Hp was analyzed using a wide-field epi-fluorescence microscope (Olympus IX73, Shinjuku, Tokyo, Japan). Analysis of the double labeling was performed by switching between the specific filter sets used for each fluorophore, DAPI for cell nuclei (blue), rhodamine for Hb (red), and AF488 for Hp (green), together with digital image documentation (Olympus DP80). The separate images for each channel (fluorophore) were merged for detailed analyses of double labeling to identify them as co-existing or not (see representative images in Fig. 2). To ensure sole detection of primary antibody binding, i.e., excluding detection of autofluorescence or of nonspecific secondary antibody binding, the detection level (threshold) for each channel was always set from sections with antibody controls (see above) and from sections from control animals that had been taken through the whole labeling protocol. Analyses and digital imaging were performed with the preset detection levels (detection intensities solely from specific labeling) for each channel. The relatively strong autofluorescence from cell bodies, mainly from RBCs, could be clearly separated from the non-cell body-associated, cell-free, and widely distributed Hb in IVH animals and together with Hp in IVH animals that received Hp (see Fig. 2).

Immunohistochemistry of Cerebellar Development and Reactive Microgliosis

To investigate the effect of IVH on the cerebellum of preterm rabbit pups, IHC labeling against the following antigens was

performed: (1) Ki67, to evaluate cellular proliferation; (2) calbindin, to evaluate Purkinje cell development and maturation; and (3) Iba1, to evaluate microglial activation. Qualitative and quantitative analysis at P0, P2, and P5 were performed. Briefly, the protocol was as follows. After antigen retrieval and rinsing in PBS, sections were incubated with primary antibodies (diluted in PBS + 5% normal goat serum, Jackson ImmunoResearch, 005-000-121) for 1 h at RT. Primary antibodies were made against rabbit Ki67 (mouse IgG anti-Ki67, Dako, Copenhagen, Denmark), calbindin (mouse IgG anti-calbindin, DBS, Pleasanton, CA), and Iba1 (rabbit IgG anti-Iba1, Biocare, Concord, CA). Sections were then rinsed in PBS (3 × 2 min). To detect the primary antibody, sections were incubated with either BrightVision rabbit/horseradish peroxidase (HRP) or BrightVision mouse/HRP (DPVR110HRP or DPVM110HRP, both from Immunogen) for 30 min at RT. Sections were then rinsed in Tris (0.05 M, pH 7.6, 3 × 2 min). To visualize the HRP conjugations, sections were incubated with a diaminobenzidine (DAB; 50 mg DAB, Sigma, dissolved in 100 ml Tris buffer, pH 7.6, 3 × 2 min) and 100 µl of hydrogen peroxide (Merck, prepared just prior to incubation) solution was added for 5 min at RT. After rinsing in Tris (3 × 2 min), hematoxylin staining of cell nuclei (Mayers HTX, Bio-Optica) was performed for 5 s, after which the sections were dehydrated and slides were then mounted with coverslips (X-Tra-Kitt, Medite, Burgdorf, Germany). Antibody specificity tests were performed on parallel sections to confirm that the visualized immunostaining was specific for the primary antibodies. In these tests, the primary antibodies were excluded from the labeling protocol (Fig. 2 in the Data supplement). Analysis and image documentation for the results of qualitative and quantitative analysis (see below) of IHC labeling were performed with a bright-field microscope (Leica DMRX), equipped with a digital camera (Leica MC120HD).

Measurement of the width (µm) of the proliferative external granular layer (EGL), as determined by Ki67-positive cells, was performed in four predefined regions. These regions were the inner and outer portions of lobule V and the inner and outer portions of lobule IX, respectively, as illustrated in Fig. 3 in the Data supplement. These regions were chosen because they represent regions with possible maturational differences in EGL proliferation and subsequent width. Measurements were performed with a bright-field microscope (Leica DMRX), using a ×40 dry objective lens. The average of the four respective measured widths was calculated for each pup.

Using the Leica Q500 image analysis system of the microscope, the areas of Iba1- and calbindin-positive stained cells were respectively determined in relation to the cerebellar white matter area and the area of the molecular layer. Thus, both positive Iba1 and calbindin staining were expressed as percentage positive area in relation to, respectively, a standardized area of the cerebellar white matter and of the

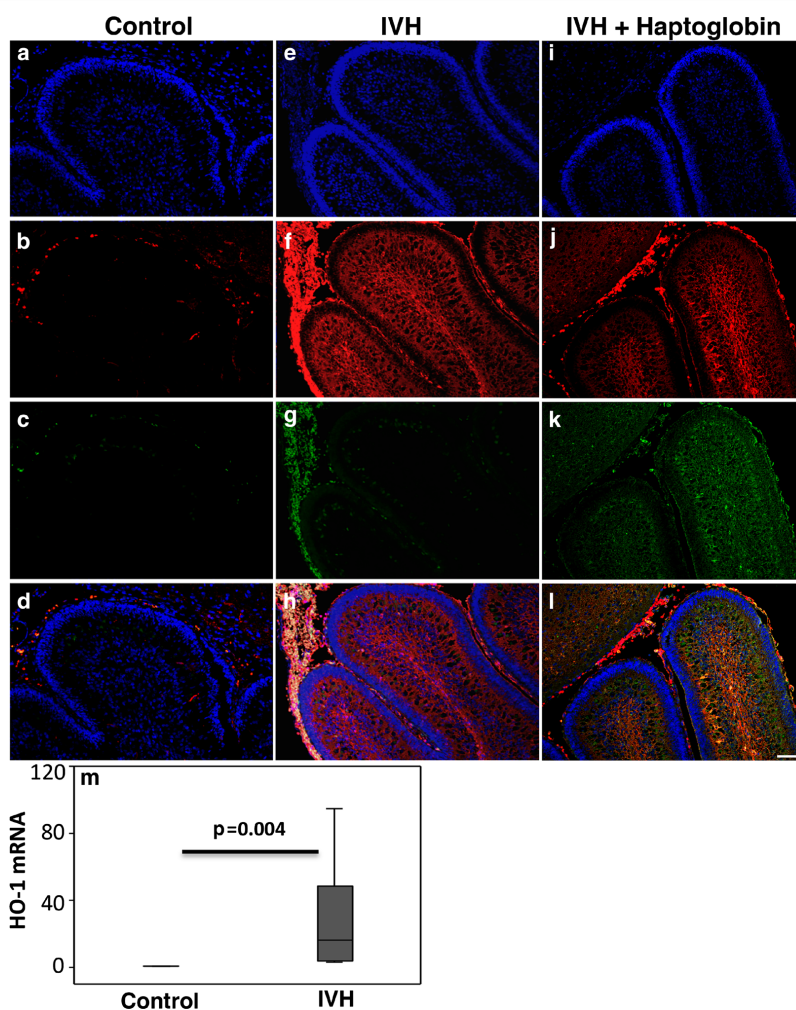


Fig. 2 Immunofluorescence labeling of Hb and the administered human Hp. Representative images are from rabbit pups at P0. Images illustrate the detected immunofluorescence labeling, performed by double immunofluorescence labeling of Hb (red) and Hp (green) together with a DAPI nuclear staining (blue), in animals with no IVH (Control), in animals with IVH (IVH), and in animals with IVH that received human Hp injections (IVH + Haptoglobin). Antibody specificity tests showed that the antibodies against Hb and human Hp bound to their true targets (see Fig. 1 in the Data supplement). **a–d** Control animal: Images **b** and **c** show the lack of Hb and Hp labeling and the autofluorescence mainly from whole erythrocytes (RBCs) restricted to the subarachnoid space and some blood vessels (**d**). **e–h** IVH: In pups with IVH, the Hb labeling (red) was extensive, widely distributed in the molecular layer and white matter and to some degree in the EGL. Whole erythrocytes in the subarachnoid space surrounding the cerebellar lobuli were also intensely labeled and gave rise to green autofluorescence (**g**), observed as yellowish in the merged image (**h**). Hb labeling intermingled with dense nuclear regions (intense DAPI staining) appears as

pink (bottom images). **i–l** IVH + Haptoglobin: **j** and **k** show immunofluorescence labeling of Hb (red) and human Hp (green) following intraventricular injection of Hp at E29. **j** shows the widespread distribution of cell-free Hb (red), corresponding to that in IVH animals (**f**), and the domination coexistence of Hp in **k** (green), primarily in the molecular layer, white matter, and the EGL as shown in the merged image (**l**). Hp labeling was scarce in the subarachnoid space (**k** and **l**), in which Hb labeling of RBCs was extensive (**j** and **l**). Thus, the cell-free Hb and Hp are clearly distinguishable from the cell body-associated Hb labeling and autofluorescence. Scale bar = 50 μ m. **m** HO-1 mRNA expression in the cerebellum was investigated at P0 following IVH. Following IVH, heme-degrading protein HO-1 mRNA was upregulated (IVH, dark gray bar, $n = 7$) as compared to the controls ($n = 5$). mRNA expression for HO-1 was normalized against GAPDH and is given as fold change. The fold change values were calculated by normalizing against samples from control pups. Results are presented as box plots displaying medians and 25th and 75th percentiles. Differences between no IVH and IVH at P0 were analyzed using the Mann–Whitney U test

molecular layer. Nonspecific background staining was taken into consideration with respect to a setup threshold.

For mRNA analysis, the rabbit pups were euthanized with intracardiac thiopental injection at P0. The brain was dissected out of the skull and cerebellar tissue collected, snap-frozen, and stored at -80°C until further analysis as described below.

RNA Isolation and Real-Time PCR

Total RNA was extracted from the cerebellar tissue of the rabbit pups using the NucleoSpin RNA/protein extraction kit as described by the manufacturer (Macherey-Nagel, Neumann-Neander, Dürren, Germany). The optical density ratio (OD at 260 nm/280 nm) of extracted RNA samples was always approximately 2.0. Reverse transcription was performed according to the manufacturer's instructions on 1 μg total RNA using iScriptTM cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). The RT² qPCR Primer Assay (primer from QIAGEN, Germantown, MD, USA) was used to quantify mRNA expression of heme oxygenase 1 (HO-1), and expression was analyzed using iTaq Universal SYBR Green Supermix (Bio-Rad). Amplification was performed as described by the manufacturer (Bio-Rad) for 40 cycles in an iCycler Thermal Cycler (Bio-Rad), and data were analyzed using iCycler iQ Optical System Software (Bio-Rad). Data were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH, primer from QIAGEN), with fold change values calculated by normalizing against control animals.

Statistics

Statistical analysis was performed with IBM SPSS Statistics version 22. Results are presented as medians (ranges) and displayed as box plots. Comparisons between unrelated groups were performed with the Mann–Whitney *U* test as appropriate. Comparisons between multiple groups were made using the Kruskal–Wallis test followed by pairwise comparison with significance values adjusted for multiple comparisons. *P* values <0.05 were considered significant.

Results

Extensive Presence of Cell-Free Hb in the Cerebellum Following IVH

Immunofluorescence labeling of Hb was evaluated at P0 and revealed extensive deposition of RBCs in the subarachnoid space surrounding the cerebellar lobuli following IVH, which was not observed in control animals (control and IVH in Fig. 2). Labeled Hb was widespread within the cerebellum and not associated

with cell bodies (IVH in Fig. 2). Extensive deposition of radially oriented cell-free Hb was observed in the deeper cerebellar layers, in the molecular layer, and in the white matter.

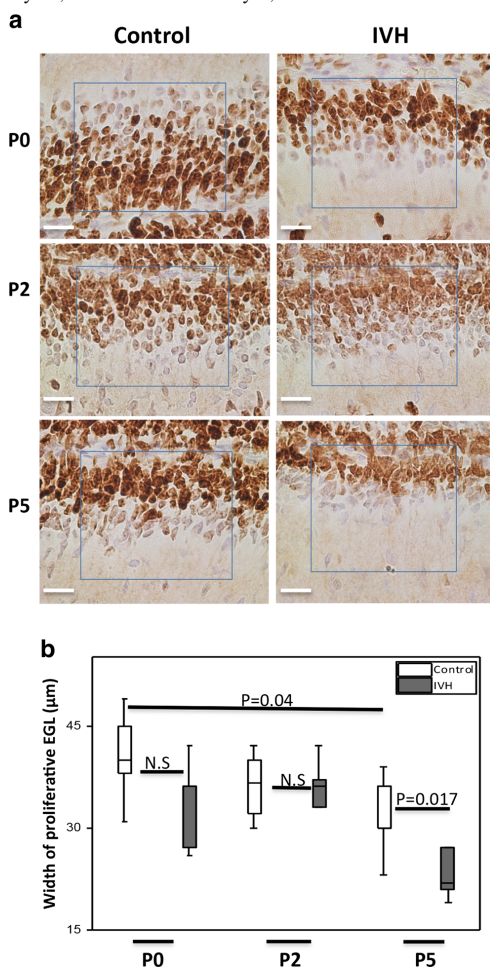
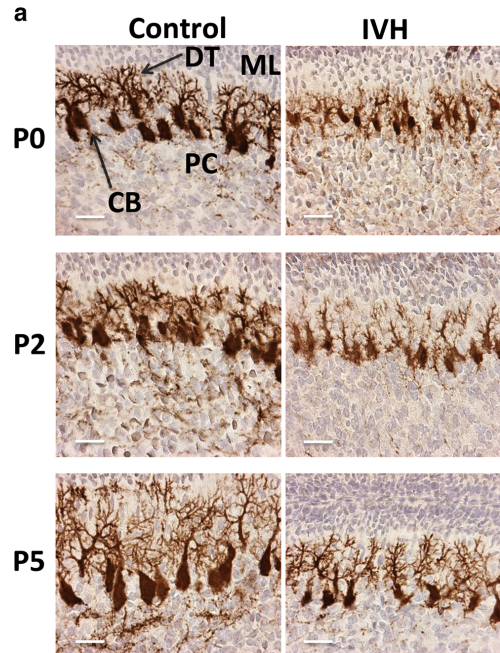


Fig. 3 Reduced width of proliferative EGL following preterm IVH. **a** Images of EGL of the developing cerebellum from which quantitative measurements were made of the proliferative width in the respective groups at the time points studied. The image shows the Ki67-positive outer portion of the EGL where proliferation of granule cell precursors occurs and the deeper portion, which hosts the differentiation of granule cell precursors to mature granule cells. Scale bar = 50 μm . **b** GCP proliferation in the outer portion of the EGL of the developing cerebellum was investigated following IVH by Ki67 staining. Measurement of the width of proliferative EGL was done in cerebellar tissue sections of both sham controls (control, white bars; *n* at P0 = 6, *n* at P2 = 6, *n* at P5 = 5) and IVH pups (dark gray bars; *n* at P0 = 5, *n* at P2 = 6, *n* at P5 = 6) at P0, P2, and P5. Results are presented as box plots displaying medians and 25th and 75th percentiles. Statistical differences between groups for respective time points were analyzed using the Mann–Whitney *U* test

Fig. 4 Impaired Purkinje cell maturation following preterm IVH. **a** Immunostaining of calbindin, a calcium-binding protein, was used as a marker of Purkinje cell development in the molecular layer of the developing cerebellum. Calbindin stains are seen as brown to dark brown. Decreased calbindin immunoreactivity was observed in IVH pups (brown) compared to controls (intense dark brown). Observation of neuronal morphology revealed smaller neuronal cell bodies and underdeveloped Purkinje dendrites in IVH pups compared to controls at postnatal time points of P0, P2, and P5. *ML* molecular layer, *PC* Purkinje cell, *DT* dendrites, *CB* cell bodies; *scale bar* = 50 μ m. **b** Grading of Purkinje cell development by measurement of percentage area of positive calbindin staining was done in cerebellar tissue sections of both control (white bars; *n* at P0 = 6, *n* at P2 = 6, *n* at P5 = 5) and IVH pups (dark gray bars; *n* at P0 = 6, *n* at P2 = 6, *n* at P5 = 6) at P0, P2, and P5, as described in “Materials and Methods.” Results are presented as box plots displaying medians and 25th and 75th percentiles. Statistical differences between groups for respective time points were analyzed using the Mann–Whitney *U* test



Relatively low amounts of cell-free Hb molecules were observed in the EGL and primarily in lobules in immediate proximity to large deposits of RBCs in the subarachnoid space.

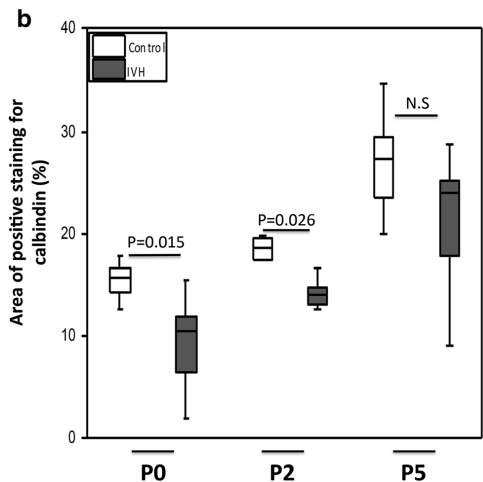
To further investigate the indicated widespread presence of cell-free Hb in P0 IVH pups shown by immunofluorescence labeling, we performed RT-PCR analysis of mRNA expression in cerebellar tissue of the major heme-degrading protein heme-oxygenase 1 (HO-1). At P0, the HO-1 mRNA expression levels were tenfold higher in IVH pups compared to controls (Fig. 2m).

EGL Proliferation Following IVH

The total width of the EGL comprises an outer proliferative portion where the granule cell precursors (GCPs) divide and a deeper portion where the granule cells differentiate [3]. The width of the outer proliferative (Ki67-positive) portion of the EGL was measured and compared between groups (Fig. 3a, b). The median (range) widths of the proliferative EGL were 36.0 (42–26), 36.0 (42–26), and 22.0 (27–19) μ m, respectively, at P0, P2, and P5 in the IVH pups and 40.0 (49–31), 36.5(42–30), and 30.0 (39–23) μ m, respectively, in the control pups. The median proliferative EGL width was significantly smaller in pups with IVH compared to control pups at P5 ($P = 0.017$) with a clear tendency at P0 ($P = 0.08$) (Fig. 3b).

Purkinje Cell Maturation Following IVH

Staining of calbindin, a calcium-binding protein, was used to evaluate Purkinje cell maturation in the molecular layer of the cerebellar cortex. IVH pups had smaller neuronal cell bodies and underdeveloped dendritic processes compared to control pups at P0, P2, and P5, respectively (Fig. 4a). Purkinje cell calbindin labeling was calculated and graded using densitometry, which showed that calbindin-labeled Purkinje cells at P0



and P2 had a significantly lower area in the IVH pups compared to controls (Fig. 4b; P0, $P = 0.015$; P2, $P = 0.026$); however, for smaller cells at P5, the differences were not statistically significant ($P = 0.247$). The smaller size in IVH animals indicated a reduced Purkinje cell differentiation and maturation in IVH animals.

Fig. 5 Microglial activation in the cerebellar white matter following preterm IVH. **a** Immunolabeling to confirm upregulation of Iba1 (seen as brown to dark brown) expression, a marker of microglial activation was used as a qualitative marker of reactive microglia cellular response in the white matter of the developing cerebellum. Increased Iba1 immunoreactivity was observed in IVH pups compared to controls at P0, P2, and P5. Observation of microglial morphology revealed an amoeboid shape with long processes in the IVH pups. Scale bar = 50 μ m. **b** Measurement of percentage area of positive Iba1 staining was done in cerebellar tissue sections of both control (white bars; n at P0 = 6, n at P2 = 6, n at P5 = 5) and IVH pups (dark gray bars; n at P0 = 5, n at P2 = 6, n at P5 = 6) at P0, P2, and P5, as described in “Materials and Methods.” Results are presented as box plots displaying medians and 25th and 75th percentiles. Statistical differences between groups for respective time points were analyzed using the Mann–Whitney U test

Microglial Response in Cerebellar White Matter Following IVH

Iba1 immunoreactivity was investigated to evaluate cerebellar white matter microglial response following IVH (Fig. 5a). At P0 and P2, IVH pups compared to control pups showed a significantly higher area of Iba1 immunoreactivity, based on cells with amoeboid morphology corresponding to activated microglia (P0, $P = 0.009$; P2, $P = 0.004$; see Fig. 5b). Microglial activation was less marked at P5 ($P = 0.247$) in both groups and did not differ significantly between groups.

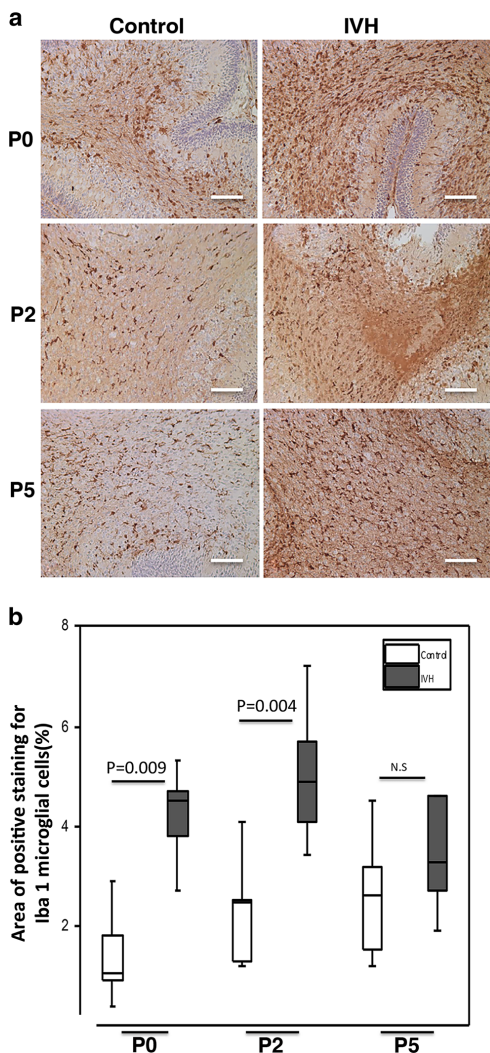
Hp Distribution Following Intraventricular Administration

At P0, the presence of Hp and its distributional relation to cell-free Hb was investigated in all groups by means of double immunofluorescence labeling (Fig. 2). Hp labeling was detected only in IVH pups that received intraventricular (human) Hp at 8 h of age. No Hp labeling was detected in control pups or in pups with IVH receiving only vehicle.

In IVH pups receiving human Hp, the Hp immunolabeling was widely distributed throughout large parts of the cerebellum. Double immunofluorescence labeling of Hp and Hb in these pups displayed a high degree of co-existence of human Hp and Hb in most regions, including the molecular layer and white matter (Fig. 2, IVH + Hp). Similar to labeling of cell-free Hb, labeling of Hp was relatively low in the EGL.

Reduced Cerebellar Damage Following Hp Administration

The group of pups receiving intraventricular administration of Hp following IVH (IVH + Hp), displayed an improved Purkinje cell maturation at P0 compared to both IVH + Vehicle pups and IVH pups (Fig. 6a–d). These findings



included both a higher intensity of calbindin immunoreactivity and relatively larger neuronal cell bodies with more developed dendritic processes (Fig. 6a–d). Results from quantification of Purkinje cell development by calbindin staining densitometry showed an increased staining in the IVH pups following intraventricular Hp administration (Fig. 6e; Control, IVH + Hp, $P = 1.00$; Control, IVH + Vehicle, $P = 0.024$).

Furthermore, Hp administration restored the arrested cell proliferative activity in the outer portion of the EGL at P0 following IVH, as shown by the width of the proliferative part of the EGL in the respective treatment groups (described in

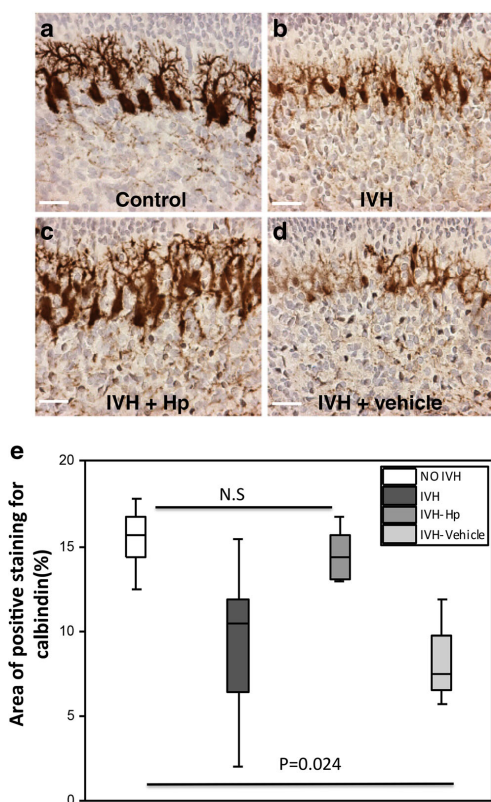


Fig. 6 Intraventricular Hp administration protects against impaired Purkinje cell development following preterm IVH. **a–d** Following intraventricular Hp administration at P0, a higher intensity of calbindin immunoreactivity, relatively larger Purkinje cell bodies, and developed dendrites were observed in the Hp-administered IVH pups as compared to pups with IVH only or vehicle-treated IVH pups. Scale bar = 50 μ m. **e** Grading of Purkinje cell development by measurement of percentage area of positive calbindin staining was done in cerebellar tissue sections at P0 of control pups (white bars, $n = 6$), IVH pups (dark gray bars, $n = 6$), and following intraventricular injection of Hp in pups with IVH (IVH + Hp, gray bars, $n = 6$) or vehicle solution (IVH + Vehicle, light gray bars, $n = 4$). Results are presented as box plots displaying medians and 25th and 75th percentiles. Differences between IVH + Hp vs. control and IVH + Vehicle vs. control were analyzed using the Kruskal–Wallis test followed by pairwise comparison with significance values adjusted for multiple comparisons

Fig. 7a–d). The median (range) widths of the proliferative EGL were 39 (48–32) μ m in the IVH + Hp pups, 30.5 (36–26) μ m in the IVH + Vehicle pups, 36.0 (42–26) μ m in the IVH pups, and 40.0 (49–31) μ m in the control pups (Fig. 7e; Control, IVH + Hp, $P = 0.93$; Control, IVH + Vehicle, $P = 0.038$).

Discussion

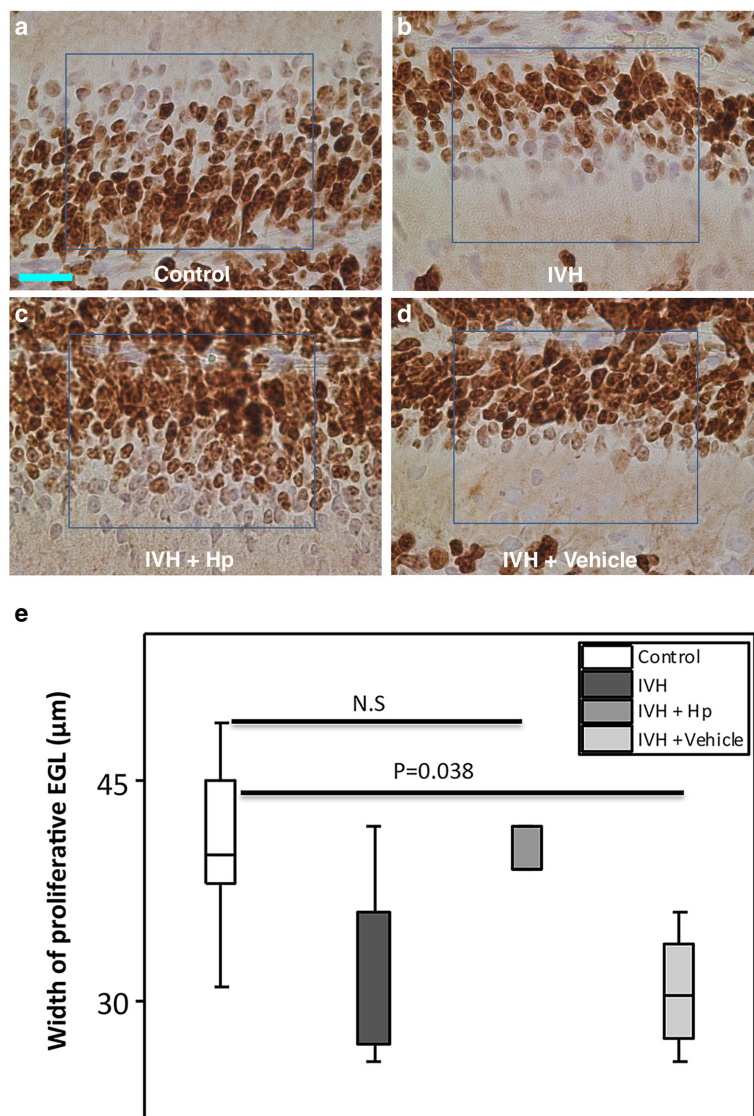
In this study, we show that IVH in the preterm rabbit pup is followed by an extensive deposition of blood products, specifically cell-free Hb, in the cerebellar cortex and white matter. This event is accompanied by a decrease in neuronal cell proliferation and a delay in Purkinje cell maturation. Intraventricular administration of the cell-free Hb scavenger Hp resulted in a high co-existence of administered Hp with cell-free Hb within the cerebellum. Furthermore, administered Hp partially reversed the cerebellar damage, indicating that cell-free Hb and its metabolites are causal in cerebellar underdevelopment. To the best of our knowledge, this work is the first animal study to evaluate cerebellar exposure to blood products and their role in cerebellar impairment following preterm IVH.

Following preterm IVH, there is a deposition of extravasated blood into the CSF of the intraventricular space. This deposition is followed by hemolysis of RBCs, leading to a release of cell-free Hb. Physiologically, cerebral CSF produced by the choroid plexus of the ventricular system passes through the fourth ventricle and enters the subarachnoid space, resulting in an immediate interface with the cortex of the developing cerebellum [27, 28]. Consequently, there is a strong physiological support for CSF containing extravasated blood reaching the cerebellum following cerebral IVH, as evidenced in this study by the visible presence of hemorrhagic CSF surrounding cerebellar tissue at termination of pups with IVH.

In the rabbit pup model, the spontaneous vessel rupture and the subsequent sequence of events leading to IVH mimics the situation in the human preterm infant quite well. It has been suggested that many of the effects observed in this model are related to the administered glycerol, including decreased proliferation leading to cerebellar hypoplasia [29]. Of importance in this study, as well as in our previous work, all pups including controls received the same dose of intraperitoneal glycerol, which rules out the possibility that the present findings in IVH pups are related to the administered glycerol.

Using Hb immunofluorescence and as demonstrated by autofluorescence, we identified an extensive deposition of RBCs and cell-free Hb in the subarachnoid space enveloping the cerebellar lobules following IVH. Cell-free Hb reached the innermost layers of the cerebellar cortex at P0 and was extensively deposited in the molecular layer and white matter of the cerebellum but to a much lesser extent in the EGL. Cell-free Hb within the EGL was basically found only in cerebellar lobules in immediate proximity to large deposits of RBCs in the subarachnoid space, possibly serving as a source of the cell-free Hb. In conjunction with the radial orientation of the Hb molecules, the high amount of cell-free Hb in the molecular layer and white matter suggests additional sources beyond the CSF in the subarachnoid space. Speculatively, the source of cell-free Hb could be via the roof of the fourth ventricle and

Fig. 7 Intraventricular Hp administration protects against reduction in width of proliferative EGL following preterm IVH. **a–d** Following intraventricular Hp administration at P0, a higher intensity of Ki67 immunoreactivity was observed in the Hp-administered IVH pups as compared to pups with IVH only or vehicle-treated IVH pups. Scale bar = 20 μ m. **e** Measurement of the width of Ki67-positive proliferative EGL was performed in cerebellar tissue sections at P0 of control pups (white bars, $n = 6$), IVH pups (dark gray bars, $n = 5$), and following intraventricular injection of Hp in pups with IVH (IVH + Hp, gray bars, $n = 5$) or vehicle solution (IVH + Vehicle, light gray bars, $n = 4$). Results are presented as box plots displaying medians and 25th and 75th percentiles. Differences between IVH + Hp vs. control and IVH + Vehicle vs. control were analyzed using the Kruskal–Wallis test followed by pairwise comparison with significance values adjusted for multiple comparisons



transfer through the cerebellar peduncles to the white matter of the cerebellum.

Cell-free Hb and its metabolites, e.g., heme and iron, are well described to act as sources of ROS and free radicals, which are causal initiators of oxidative damage to cells and tissues [30]. We have previously shown that cell-free Hb and its metabolites, i.e., methemoglobin and heme, are potent inducers of pro-inflammatory pathways in choroid plexus

epithelium and in astrocytes [17–19]. The extensive presence of cell-free Hb in the cerebellar white matter following IVH in this study was accompanied by clear signs of microglial activation in corresponding white matter regions, marked by increased expression of Iba1 antigen and an activated morphology in the IVH group (Fig. 5). This result suggests that deposited cell-free Hb may induce a microglial pro-inflammatory response with possible

adverse effects on immature oligodendrocyte proliferation and maturation and subsequent cerebellar white matter damage. In the current study, using immunofluorescence and immunohistochemistry, we could not distinguish between different forms of oxidized Hb, e.g., oxyHb and metHb, and thus cannot conclude whether the effects observed are caused by oxyHb, metHb, or the downstream metabolites heme and iron.

Our finding that cell-free Hb is extensively deposited in the molecular layer of the cerebellum is a cause for concern because this layer constitutes the environment for Purkinje cell maturation. The Purkinje cells are intrinsically sensitive to oxidative stress and essential for establishing the cerebellar circuitry, which is vital for impulse transmission in the cerebellum [31–34]. In addition, mature Purkinje cells also play a vital role in the development of the EGL by sourcing GCPs with sonic hedgehog protein, an important mitotic growth factor vital to their proliferation [35, 36]. Consequently, exposure of the molecular layer to cell-free Hb not only will have neurotoxic effects on Purkinje cells but also will further impair the development of the EGL. The EGL of the developing cerebellum serves as a germinal center where GCPs proliferate and subsequently differentiate into mature granule cells. Granule cells are important for the structural integrity of the cerebellum; in addition, during their migration to form the granular layer, they transmit certain excitatory signals needed for the differentiation and maturation of the Purkinje cells. Thus, exposure of the developing cerebellum to cell-free Hb may lead to damaging effects not only to the cellular architecture but also to the functional integrity of the cerebellum, subsequently causing cerebellar underdevelopment.

To evaluate the possible effects of impaired Purkinje cell support and direct exposure to the hemorrhage, we performed metric analysis of Ki67 staining to evaluate EGL cell proliferation and thus pathological cellular senescence. Cellular senescence in this context can be seen as a process by which damage to tissue causes a decrease in metabolism leading to arrest of cell proliferation and recruitment of phagocytic immune cells to help in tissue renewal [37]. Measurements of the EGL (Fig. 3a) showed that IVH caused a significant decrease in the width of the proliferative portion of the EGL at P0 and P5 (Fig. 3b). This result is a clear indication that IVH-related processes cause impairment of the proliferative activity of the EGL. The postnatal time points P0 to P5 studied in the preterm rabbit pup correspond to the gestational ages of 25 to 35 weeks in humans, a period characterized by intense cell proliferation in the outer portion of the EGL [38]. In the human preterm infant, the width of the proliferative EGL decreases from 30 gestational weeks onwards as the GCPs mature into granule cells and leave the EGL to form the internal granular layer [3]. This timing corresponds well to our observations in the rabbit pup with EGL proliferative width in control pups showing a decrease in width from P0 to P5 (Fig. 3b).

Cell-free Hb may cause damage to the cerebellum in a number of different ways. Following hemolysis, release of excess cell-free Hb may lead to the formation of heme and free iron, increasing the concentration of redox-active iron in the extracellular environment. Both heme and free iron have a pro-oxidative damaging effect on cells, and iron overload has been reported to cause cerebral damage following IVH [39, 40]. Indeed, reduction in iron overload attenuated development of hydrocephalus and brain damage in a rodent model of neonatal germinal matrix hemorrhage [41]. In addition to its redox-related effects, cell-free Hb also acts as a redox-active damage-associated molecular pattern (known as DAMP) molecule that perturbs the innate immune homeostasis by triggering Toll-like receptor signal transduction pathways and causing pro-inflammatory damage to cells [42–44]. In this study, we investigated the causal importance of cell-free Hb in the impairment of Purkinje cell maturation and in the arrest of EGL cell proliferation by administering the Hb-scavenging protein Hp intraventricularly following detection of IVH. Hp binds to cell-free Hb, forming an inert Hb–Hp complex, which then channels the Hb molecules for intracellular degradation via CD163-mediated endocytosis [45, 46]. Intracellularly, the enzyme HO-1 breaks down heme to bilirubin and CO, both of which have antioxidant and vasodilatory benefits [47]. By forming a tight complex with cell-free Hb, Hp stabilizes and shields heme iron within the hydrophobic pocket of Hb, thereby preventing its cytotoxic and pro-oxidative effect [48]. The removal of cell-free Hb from the extracellular environment through its complex formation with Hp could thus reduce interaction with signal-transducing receptors of cells in the brain innate immune system and reduce exposure to excess iron and to heme-induced toxicity.

A neuroprotective role of induced endogenous Hp following intracerebral hemorrhage has been documented [49]. The induction of Hp was necessary because of very low levels of endogenous Hp in the human brain. In a previous study, the resting state capacity of the intrathecal Hb–Hp complex clearance was found to be 50,000-fold lower than that in the circulation in the adult. The system was quickly saturated during SAH with a residual inability to deal with cell-free Hb, clearly indicating an insufficient Hb scavenging capacity within the brain [50]. In view of this, we administered human Hp intraventricularly, which resulted in an extensive presence of Hp in the cerebellum. Hp was not detected in animals that did not receive exogenous Hp. The Hp labeling was specific for the administered human Hp, i.e., completely absent in sham-injected IVH pups as in IVH and control pups, thus excluding endogenous Hp as a source of the positive Hp labeling.

Our double immunofluorescence of Hb and Hp showed that the injected Hp reaches the same cerebellar areas as cell-free Hb and that the two are extensively co-localized in these regions. Hb and administered Hp co-existed in several regions of the cerebellum, mainly within the molecular layer

and white matter and to a lesser degree in the EGL. Congruent with the anatomical co-existence of Hp and Hb, results showed that Hp administration partially reduced the Purkinje cell maturational arrest caused by IVH, represented by calbindin immunoreactivity showing a higher intensity of labeling, relatively larger cell bodies, and more extensive dendritic processes in pups receiving Hp as compared to the other IVH groups. Furthermore, Hp administration counteracted the decreased development of the proliferative region of the EGL following IVH and increased the proliferative width almost to the level of the control pups.

Conclusion

In this study, we showed that IVH in the preterm rabbit pup is followed by an extensive deposition of cell-free Hb in cerebellar cell layers and white matter. This exposure to cell-free Hb was associated with microglial activation, an arrest in neuronal cell proliferation, and a delayed Purkinje cell maturation. Intraventricular administration of the cell-free Hb scavenger Hp partially blocked these effects, suggesting that cell-free Hb and its downstream metabolites are causal in cerebellar impairment following IVH. In terms of future clinical application, these results suggest that removal or scavenging of Hb metabolites following IVH, for instance by administered Hp, may reduce subsequent cerebellar impairment.

BSA, bovine serum albumin; EGL, external granular layer; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GCP, granular cell precursor; Hb, hemoglobin; Hp, haptoglobin; IVH, intraventricular hemorrhage; PBS, phosphate buffer saline; PFA, paraformaldehyde; SAH, subarachnoid hemorrhage

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Compliance with Ethical Standards

Conflict of Interest All authors declare that they have no conflict of interest.

Ethical Approval All applicable national and institutional guidelines for the care and use of animals were followed.

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References

1. Ballabh P. Intraventricular hemorrhage in premature infants: mechanism of disease. *Pediatr Res*. 2010;67:1–8.
2. Schmahmann JD, Smith EE, Eichler FS, Filley CM. Cerebral white matter: neuroanatomy, clinical neurology, and neurobehavioral correlates. *Ann N Y Acad Sci*. 2008;1142:266–309.
3. Volpe JJ. Cerebellum of the premature infant: rapidly developing, vulnerable and clinically important. *J Child Neurol*. 2009;24:1085–104.
4. Parker J, Mitchell A, Kalpakidou A, Walshe M, Jung HY, Nosarti C, et al. Cerebellar growth and behavioural & neuropsychological outcome in preterm adolescents. *Brain*. 2008;131:1344–51.
5. Limperopoulos C, Chinaryan G, Sullivan N, Guizard N, Robertson RL, Du Plessis AJ. Injury to the premature cerebellum: outcome is related to remote cortical development. *Cereb Cortex*. 2014;24:728–36.
6. Kitai Y, Hirai S, Ohmura K, Ogura K, Arai H. Cerebellar injury in preterm children with cerebral palsy after intraventricular hemorrhage: prevalence and relationship to functional outcomes. *Brain and Development*. 2015;37:758–63.
7. Tam EW, Miller SP, Studolme C, Chau V, Glidden D, Poskitt KJ, et al. Differential effects of intraventricular hemorrhage and white matter injury on preterm cerebellar growth. *J Pediatr*. 2011;158:366–71.
8. Limperopoulos C, Soul JS, Gauvreau K, Huppi PS, Warfield SK, Bassan H, et al. Late gestation cerebellar growth is rapid and impeded by premature birth. *Pediatrics*. 2005a;115:688–95.
9. Messerschmidt A, Prayer D, Brugger PC, Boltshauser E, Zoder G, Sterniste W, et al. Preterm birth and disruptive cerebellar development: assessment of perinatal risk factors. *Eur J Paediatr Neurol*. 2008;12:455–60.
10. Limperopoulos CS, Haidar H, Huppi PS, Bassan H, Warfield SK, Robertson RL, et al. Impaired trophic interactions between the cerebellum and the cerebrum among preterm infants. *Pediatrics*. 2005b;116:844–50.
11. Van Kooij BJ, Benders MJ, Anbeek P, Van Haastert IC, De Vries LS, Groenendaal F. Cerebellar volume and proton magnetic resonance spectroscopy at term, and neurodevelopment at 2 years of age in preterm infants. *Dev Med Child Neurol*. 2012;54:260–6.
12. Tam EW. Potential mechanisms of cerebellar hypoplasia in prematurity. *Neuroradiology*. 2013;55(Suppl 2):41–6.
13. Nosarti C, Giouroukou E, Micali N, Rifkin L, Morris RG, Murray RM. Impaired executive functioning in young adults born very preterm. *J Int Neuropsychol Soc*. 2007;13:571–81.
14. Indredavik MS, Vik T, Evensen KA, Skranes J, Taraldsen G, Brubakk AM. Perinatal risk and psychiatric outcome in adolescents born preterm with very low birth weight or term small for gestational age. *J Dev Behav Pediatr*. 2010;31:286–94.
15. Lee JY, Keep RF, He Y, Sagher O, Hua Y, Xi G. Hemoglobin and iron handling in brain after subarachnoid hemorrhage and the effect of deferoxamine on early brain injury. *J Cereb Blood Flow Metab*. 2010;30:1793–803.
16. Lok J, Leung W, Murphy S, Butler W, Noviski N, Lo EH. Intracranial hemorrhage: mechanisms of secondary brain injury. *Acta Neurochir Suppl*. 2011;111:63–9.
17. Gram M, Sveinsdóttir S, Ruscher K, Hansson SR, Cinthio M, Åkerström B, et al. Hemoglobin induces inflammation after preterm intraventricular hemorrhage by methemoglobin formation. *J Neuroinflammation*. 2013;10:100.
18. Gram M, Sveinsdóttir S, Cinthio M, Sveinsdóttir K, Hansson SR, Mörgelin M, et al. Extracellular hemoglobin—mediator of inflammation and cell death in the choroid plexus following preterm intraventricular hemorrhage. *J Neuroinflammation*. 2014;11:200.

19. Sveinsdóttir S, Gram M, Cinthio M, Sveinsdóttir K, Mörgelin M, Ley D. Altered expression of aquaporin 1 and 5 in the choroid plexus following preterm intraventricular hemorrhage. *Dev Neurosci*. 2014;36:542–51.
20. Ley D, Romantsik O, Vallius S, Sveinsdóttir K, Sveinsdóttir S, Agyemang AA, et al. High presence of extracellular hemoglobin in the periventricular white matter following preterm intraventricular hemorrhage. *Front Physiol*. 2016;7:330.
21. Kumar S, Bandyopadhyay U. Free heme toxicity and its detoxification systems in human. *Toxicol Lett*. 2005;157:175–88.
22. Olsson MG, Allhom M, Bulow L, Hansson SR, Ley D, Olsson ML, et al. Pathological conditions involving extracellular hemoglobin: molecular mechanisms, clinical significance, and novel therapeutic opportunities for alpha (1)-microglobulin. *Antioxid Redox Signal*. 2012;17:813–46.
23. Quayle IK. Extracellular hemoglobin: the case of a friend turned foe. *Front Physiol*. 2015;6:96.
24. Sveinsdóttir S, Cinthio M, Ley D. High-frequency ultrasound in the evaluation of cerebral intraventricular haemorrhage in preterm rabbit pups. *Ultrasound Med Biol*. 2012;38:423–31.
25. Georgiadis P, Xu H, Chua C, Hu F, Collins L, Huynh C, et al. Characterization of acute brain injuries and neurobehavioral profiles in a rabbit model of germinal matrix hemorrhage. *Stroke*. 2008;39:3378–88.
26. Chua CO, Chahboune H, Braun A, Dummula K, Chua CE, Yu J, Ungvari Z, et al. Consequences of intraventricular hemorrhage in a rabbit pup model. *Stroke*. 2009;40:3369–77.
27. Ballabh P. Pathogenesis and prevention of intraventricular hemorrhage. *Clin Perinatol*. 2014;41:47–67.
28. Sakka L, Coll G, Chazal J. Anatomy and physiology of cerebrospinal fluid. *Eur Ann Otorhinolaryngol Head Neck Dis*. 2011;128:309–16.
29. Traudt CM, Mcpherson RJ, Studholme C, Millen KJ, Juul SE. Systemic glycerol decreases neonatal rabbit brain and cerebellar growth independent of intraventricular hemorrhage. *Pediatr Res*. 2014;75:389–94.
30. Khaket TP, Ahmad R. Biochemical studies on hemoglobin modified with reactive oxygen species (ROS). *Appl Biochem Biotechnol*. 2011;164:1422–30.
31. Lopez IA, Acuna D, Beltran-Parral L, Lopez IE, Amarnani A, Cortes M, et al. Evidence for oxidative stress in the developing cerebellum of the rat after chronic mild carbon monoxide exposure (0.0025% in air). *BMC Neurosci*. 2009;10:53.
32. Hsieh JY, Ulrich B, Issa FA, Wan J, Papazian DM. Rapid development of Purkinje cell excitability, functional cerebellar circuit, and afferent sensory input to cerebellum in zebrafish. *Front Neural Circuits*. 2014;8:147.
33. White JJ, Arancillo M, Stay TL, George-Jones NA, Levy SL, Heck DH, et al. Cerebellar zonal patterning relies on Purkinje cell neurotransmission. *J Neurosci*. 2014;34:8231–45.
34. Sillitoe RV. Mossy fibers terminate directly within Purkinje cell zones during mouse development. *Cerebellum*. 2016;15:14–7.
35. Carletti B, Rossi F. Neurogenesis in the cerebellum. *Neuroscientist*. 2008;14:91–100.
36. De Luca A, Cerrato V, Fuca E, Parmigiani E, Buffo A, Leto K. Sonic hedgehog patterning during cerebellar development. *Cell Mol Life Sci*. 2016;73:291–303.
37. Munoz-Espin D, Serrano M. Cellular senescence: from physiology to pathology. *Nat Rev Mol Cell Biol*. 2014;15:482–96.
38. Clancy B, Finlay BL, Darlington RB, Anand KJ. Extrapolating brain development from experimental species to humans. *Neurotoxicology*. 2007;28:931–7.
39. Chen Z, Gao C, Hua Y, Keep RF, Muraszko K, Xi G. The role of iron in brain injury after intraventricular hemorrhage. *Stroke*. 2011;42(2):465–70.
40. Strahle JM, Garton T, Bazzi AA, Kilaru H, Garton HJL, Maher CO, et al. Role of hemoglobin and iron in hydrocephalus after neonatal intraventricular hemorrhage. *Neurosurgery*. 2014;75(6):696–706.
41. Guo J, Chen Q, Tang J, Zhang J, Tao Y, Li L, et al. Minocycline-induced attenuation of iron overload and brain injury after experimental germinal matrix hemorrhage. *Brain Res*. 2015;1594:115–24.
42. Ding JL, Lee SK. A perspective on the role of extracellular hemoglobin on the innate immune system. *DNA Cell Biol*. 2013;32:36–40.
43. Gladwin MT, Ofori-Acquah SF. Erythroid DAMPS drive inflammation SCD. *Blood*. 2014;123(24):3689–90.
44. Wang YC, Zhou Y, Fang H, Lin S, Wang PF, Xiong RP, et al. Toll-like receptor 2/4 heterodimer mediates inflammatory injury in intracerebral hemorrhage. *Ann Neurol*. 2014;75:876–89.
45. Abraham NG, Drummond G. CD163-mediated hemoglobin-heme uptake activates macrophage HO-1, providing an anti-inflammatory function. *Circ Res*. 2006;99:911–4.
46. Chintagari NR, Nguyen J, Belcher JD, Vercellotti GM, Alayash AI. Haptoglobin attenuates hemoglobin-induced heme oxygenase-1 in renal proximal tubule cells and kidneys of a mouse model of sickle cell disease. *Blood Cells Mol Dis*. 2015;54:302–6.
47. Ryter SW, Morse D, Choi AM. Carbon monoxide and bilirubin: potential therapies for pulmonary/vascular injury and disease. *Am J Respir Cell Mol Biol*. 2007;36:175–82.
48. Yang F, Haile DJ, Berger FG, Herbert DC, Van Beveren E, Ghio AJ. Haptoglobin reduces lung injury associated with exposure to blood. *Am J Physiol Lung Cell Mol Physiol*. 2003;284:402–9.
49. Zhao X, Song S, Sun G, Strong R, Zhang J, Grotta JC, Aronowski J. Neuroprotective role of haptoglobin after intracerebral hemorrhage. *J Neurosci*. 2009;29(50):15819–27.
50. Galea J, Cruickshank G, Teeling JL, Boche D, Garland P, Perry VH, et al. The intrathecal CD163-haptoglobin-hemoglobin scavenging system in subarachnoid hemorrhage. *J Neurochem*. 2012;121:785–92.

Paper III



Relation of Retinopathy of Prematurity to Brain Volumes at Term Equivalent Age and Developmental Outcome at 2 Years of Corrected Age in Very Preterm Infants

Kristbjörg Sveinsdóttir^a David Ley^a Holger Hövel^b Vineta Fellman^{a,c}
Petra S. Hüppi^d Lois E.H. Smith^e Ann Hellström^f Ingrid Hansen Pupp^a

^aDivision of Pediatrics, Department of Clinical Sciences, Skåne University Hospital, Lund University, Lund, Sweden; ^bDivision of Pediatrics, Department of Clinical Sciences, Central Hospital Kristianstad, Lund, Sweden; ^cChildren's Hospital, University of Helsinki, Helsinki, Finland; ^dDivision of Development and Growth, Department of Pediatrics, University Hospital of Geneva, Geneva, Switzerland; ^eDepartment of Ophthalmology, Boston Children's Hospital, Harvard Medical School, Boston, MA, USA; ^fSahlgrenska Center for Pediatric Ophthalmology Research, Institute of Neuroscience and Physiology, University of Gothenburg, Gothenburg, Sweden

Keywords

Brain volume · Developmental outcome · Magnetic resonance imaging · Mental developmental index · Psychomotor developmental index · Preterm birth · Retinopathy of prematurity

Abstract

Background: Retinopathy of prematurity (ROP) is a major complication of preterm birth and has been associated with later visual and nonvisual impairments. **Objectives:** To evaluate relationships between any stage of ROP, brain volumes, and developmental outcomes. **Methods:** This study included 52 very preterm infants (gestational age [mean ± SD]: 26.4 ± 1.9 weeks). Total brain, gray matter, unmyelinated white matter (UWMV), and cerebellar volumes were estimated in 51 out of 52 infants by magnetic resonance imaging at term-equivalent age. Bayley Scales of Infant Development were used to assess developmental outcomes in 49 out of 52 in-

fants at a mean corrected age of 24.6 months. **Results:** Nineteen out of 52 infants developed any stage of ROP. Infants with ROP had a lower median (IQR) UWMV (173 [156–181] vs. 204 [186–216] mL, $p < 0.001$) and cerebellar volume (18.3 [16.5–20] vs. 22.3 [20.3–24.7] mL, $p < 0.001$) than infants without ROP. They also had a lower median (IQR) mental developmental index (72 [56–83] vs. 100 [88–104], $p < 0.001$) and a lower psychomotor developmental index (80 [60–85] vs. 92 [81–103], $p = 0.002$). Brain volumes and developmental outcomes did not differ among infants with different stages of ROP. **Conclusion:** Any stage of ROP in preterm infants was associated with a reduced brain volume and an impaired developmental outcome. These results suggest that common pathways may lead to impaired neural and neurovascular development in the brain and retina and that all stages of ROP may be considered in future studies on ROP and development.

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Introduction

With the increasing survival of extremely preterm infants, a proportionately greater number of infants are affected by major complications of prematurity, where neurodevelopmental disability and visual problems are among the most common [1, 2]. Preterm birth coincides with a critical period of brain development but also with that of vascular development [3].

The retina is incompletely developed at preterm birth. Retinopathy of prematurity (ROP) is considered to be a consequence of a primary arrest of retinal vascularization and a neurovascular disease involving both vascular and neural components [4, 5]. Postnatal head growth retardation can be regarded as a proxy of brain growth and coincides with suppression of retinal vascular growth and with low levels of IGF-1, an important vascular and neural growth factor [6]. These events can be further related to the development of severe ROP and low brain volumes at term-equivalent age [6, 7].

Preterm infants who develop severe ROP appear to be at an increased risk for visual [2] and nonvisual neurodevelopmental comorbidities and delayed white matter maturation as estimated by magnetic resonance imaging (MRI) at an early postnatal age and at term-equivalent age [8–11]. These findings suggest the presence of common mechanisms in the development of these complications [6].

Although ROP is defined according to different stages, the disease is a continuous process of impaired vascular and neuronal retinal development. Therefore, our aim in this study was to evaluate the relationship between the presence of any stage of ROP, brain growth, and later developmental outcomes at 2 years of corrected age.

Materials and Methods

Study Population

The original prospective study included 64 very preterm infants born in the neonatal intensive care unit (Lund, Sweden) between January 2005 and May 2007, where IGF-1 concentrations from birth until term age in relation to growth, MRI-estimated brain volume, and developmental outcome were evaluated [7, 12, 13]. Fifty-two infants completed the study until term age, 51 out of 52 underwent MRI at term age, and 49 out of 52 received a follow-up examination at 2 years of corrected age. Inclusion criteria were: gestational age <31 weeks, absence of major congenital anomalies, and written informed parental consent. Of the 64 recruited infants, 9 did not survive until term age and the parents of 3 infants chose to leave the study.

This study was approved by the regional ethical review board of Lund, Sweden, and adhered to the tenets of the Declaration of Helsinki. All pregnancies were dated by ultrasound at 17–18 weeks of gestation.

Clinical Data

The weight standard deviation score at birth was calculated from a Scandinavian intrauterine growth curve based on fetal weights estimated by ultrasound [14]. Cumulative doses (mg/kg) of administered hydrocortisone and betamethasone were registered until postmenstrual age (PMA) 35 weeks. Total steroid exposure was estimated by converting the betamethasone dosage into hydrocortisone equivalents (1:40). Bronchopulmonary dysplasia was defined as a requirement for supplemental oxygen at PMA 36 weeks. Septicemia was defined as the presence of a positive blood culture and concomitant increased levels of C-reactive protein.

Nutritional Regime and Calculation of Intake

The nutritional strategy used for the infants has been described previously [7] and was based on individualized enteral nutrition using maternal or donor breast milk (fortified if required) and additional parenteral nutrition, initiated as soon as possible after birth. Maternal and donor breast milk were analyzed weekly for protein and energy contents, and enteral and parenteral daily intakes of protein (g/kg/day) and energy (kcal/kg/day) were prospectively calculated from birth until at least PMA 35 weeks.

Cerebral Ultrasound

Cerebral ultrasounds were performed on days 1, 3, and 7, at 3 and 6 weeks of age, and at term. Severe intracranial hemorrhage was defined as the presence of IVH grade III or parenchymal hemorrhage. White matter damage was defined as the presence of periventricular echodensities or cysts that persisted for >7 days. Severe brain damage was defined as severe intracranial hemorrhage and/or white matter damage.

ROP Examination

ROP screening followed the Swedish national protocol and began at 5–6 weeks of age, but not before PMA 31 weeks. The infants underwent retinal examinations through dilated pupils biweekly to once weekly depending on ROP severity until either the retina was fully vascularized or the condition was considered stable. ROP was classified according to the International Classification of Retinopathy of Prematurity [15], and treatment followed the recommendations of the Early Treatment for Retinopathy of Prematurity Cooperative Group [16].

Magnetic Resonance Imaging

MRI was performed on a 3-T Siemens Magnetom Allegra head scanner (Siemens AG Medical Solutions, Erlangen, Germany) in 51 out of 52 infants at term age (mean \pm SD: 40.1 \pm 0.6 gestational weeks). The protocol for image acquisition and image processing has previously been described in detail [7]. We calculated the total brain volume (TBV), gray matter volume (GMV), and unmyelinated white matter volume (UWMV) in 46 infants and the cerebellar volume in 51 infants.

Assessment at 2 Years of Corrected Age

A psychologist assessed developmental outcomes in 49 out of 52 infants at a mean (\pm SD) corrected age of 24.6 (\pm 0.8) months by means of the Bayley Scales of Infant Development (BSID-II) with 2 different index scales, i.e., the MDI and the PDI, as previously described [13].

Table 1. Clinical characteristics according to the presence of retinopathy of prematurity

	No ROP (<i>n</i> = 33)	ROP (<i>n</i> = 19)	<i>p</i> value
Gestational age, weeks	27.4 (24.3 to 30.6)	25.0 (23.0 to 27.1)	<0.001
Birth weight, g	970 (592 to 1,716)	634 (348 to 854)	<0.001
SDS weight at birth	-0.7 (-4.4 to 0.8)	-1.8 (-4.7 to 0.6)	0.1
Apgar score <7 at 5 min	11 (33)	10 (53)	0.172
Male/female ratio	15/18 (45%/55%)	10/9 (53%/47%)	0.62
Severe brain damage ^a	2 (6)	3 (16)	0.342
Septicemia	7 (21)	12 (63)	0.002
Total steroid intake ^b , mg/kg	0 (0 to 112)	34 (0 to 105)	<0.001
Bronchopulmonary dysplasia	22 (67)	16 (84)	0.206
Energy intake ^c , kcal/kg/day	120 (104 to 140)	121 (93 to 134)	0.653
Protein intake ^c , g/kg/day	3.2 (2.6 to 3.7)	3.0 (2.6 to 3.6)	0.082

Values are presented as medians (range) or numbers (%). The total number of patients was 52. SDS, standard deviation score. ^a Intraventricular hemorrhage grade III, parenchymal hemorrhage, and/or white matter damage as defined by a cerebral ultrasound. ^b Calculated hydrocortisone equivalents from birth until postmenstrual age 35 weeks. ^c From birth until postmenstrual age 35 weeks.

Statistical Analysis

Statistical analysis was performed with SPSS 23 for Microsoft Windows (IBM, Armonk, NY, USA). $p < 0.05$ was considered statistically significant. Univariate analyses of differences between groups were assessed with the Mann-Whitney U test or the χ^2 test as appropriate. Correlations between continuous variables were evaluated with the Spearman rank correlation coefficient.

Adjustment for other variables was performed with multiple linear regression analysis. In all models GA, birth weight (BW), and gender were included as independent variables. Additionally, all variables exhibiting significant univariate associations with the respective outcome variables (Table 2) were entered into the multivariate model as independent variables. Thus, independent variables entered into the analysis for cerebellar volume and UWMV, respectively, were: GA (days), BW, gender, septicemia, and total steroid intake (hydrocortisone equivalents in mg/kg) from birth until PMA 35 weeks. The independent variables entered into the analysis for MDI were: GA, BW, gender, Apgar score <7 at 5 min, and total steroid intake from birth until PMA 35 weeks. The independent variables entered into the analysis for PDI were: GA (days), BW, gender, and severe brain damage.

Results

Clinical Characteristics and Development of ROP

Table 1 presents the clinical characteristics of infants with and without ROP. Infants with any ROP ($n = 19$) had a lower GA, a lower BW, a higher frequency of septicemia, and a higher total steroid intake compared to infants without ROP.

Clinical Characteristics in Relation to Cerebellar Volume, UWMV, MDI, and PDI

Table 2 presents relationships between clinical characteristics and cerebellar volume, UWMV, MDI, and PDI, respectively. GA correlated with cerebellar volume ($r_s = 0.56$, $p < 0.001$), UWMV ($r_s = 0.71$, $p < 0.001$), and both MDI ($r_s = 0.46$, $p = 0.001$) and PDI ($r_s = 0.34$, $p = 0.015$). BW correlated with cerebellar volume ($r_s = 0.71$, $p < 0.001$), UWMV ($r_s = 0.82$, $p < 0.001$), and MDI ($r_s = 0.41$, $p = 0.004$). Apgar score <7 at 5 min was associated with a lower MDI ($p = 0.047$), and severe brain damage was associated with a lower PDI ($p = 0.025$). Any septicemia was associated with cerebellar volume ($p = 0.022$) and UWMV ($p = 0.021$). A higher total steroid intake correlated with a lower cerebellar volume ($r_s = -0.45$, $p = 0.001$), UWMV ($r_s = -0.50$, $p < 0.001$), and MDI ($r_s = -0.39$, $p = 0.006$).

Stages of ROP in Relation to Brain Volume and Neurodevelopmental Outcome

Out of 52 infants, 33 had no ROP, 9 had ROP stage 1 or 2, and 10 had ROP stage 3 (9 of these 10 infants received laser treatment). None of the infants had ROP >stage 3.

Infants with ROP stage 1 or 2 had a lower mean TBV, GMV, UWMV, cerebellar volume, MDI, and PDI compared to infants without ROP ($p = 0.001$, $p = 0.007$, $p < 0.001$, $p < 0.001$, $p = 0.008$, and $p = 0.024$, respectively). Infants with treated ROP had lower TBV, UWMV, cerebellar volume, and MDI ($p = 0.03$, $p = 0.002$, $p = 0.005$, and $p = 0.002$), whereas no significant difference could be shown for GMV or PDI, as compared to infants without ROP.

Table 2. Relationships between clinical characteristics and cerebellar volume and unmyelinated white matter volume at a postmenstrual age of 40 weeks and MDI and PDI at 2 years of corrected age

	Cerebellar volume <i>p</i> value	Unmyelinated white matter volume <i>p</i> value	MDI <i>p</i> value	PDI <i>p</i> value
Gestational age (days)	<0.001	<0.001	0.001	0.015
Birth weight (g)	<0.001	<0.001	0.004	0.191
Apgar score <7 at 5 min	0.992	0.78	0.047	0.344
Male/female ratio	0.178	0.379	0.312	0.13
Severe brain damage ^a	0.298	0.9	0.111	0.025
Septicemia	0.022	0.021	0.199	0.294
Total steroid intake ^b (mg/kg)	0.001	<0.001	0.006	0.079
Bronchopulmonary dysplasia	0.063	0.076	0.362	0.656
Energy intake ^c (kcal/kg/day)	0.72	0.723	0.961	0.787
Protein intake ^c (g/kg/day)	0.138	0.907	0.496	0.957
Maternal educational level ^d	–	–	0.099	0.352
Paternal educational level ^d	–	–	0.131	0.223

The total number of patients was 52. MDI, Mental Developmental Index; PDI, Psychomotor Developmental Index. ^a Intraventricular hemorrhage grade III, periventricular hemorrhagic infarction, and/or white matter disease as defined by a cerebral ultrasound. ^b Calculated hydrocortisone equivalents from birth until postmenstrual age 35 weeks. ^c From birth until postmenstrual age 35 weeks. ^d University degree vs. no university degree.

Table 3. Brain volumes at a postmenstrual age of 40 weeks and Mental Developmental Index and Psychomotor Developmental Index at 2 years of corrected age in infants with any ROP or no ROP

	Any ROP ^a	No ROP ^b	<i>p</i> value
Total brain volume, mL	371 (329–390)	416 (377–445)	<0.001
Gray matter volume, mL	193 (161–201)	204 (186–231)	0.054
Unmyelinated white matter volume, mL	173 (156–181)	204 (186–216)	<0.001
Cerebellar volume, mL	18.3 (16.5–20)	22.3 (20.3–24.7)	<0.001
Mental Developmental Index	72 (56–83)	100 (88–104)	<0.001
Psychomotor Developmental Index	80 (60–85)	92 (81–103)	0.002

Values are presented as medians (IQR). ROP, retinopathy of prematurity. ^a *n* = 19. ^b *n* = 32.

The mean values of brain volumes and developmental outcomes did not differ between infants with ROP (stages 1–3) and those with treated ROP.

Any ROP in Relation to Brain Volumes and Developmental Outcome

Table 3 presents the median (IQR) values of brain volumes (TBV, GMV, UWMV, and cerebellar volume) and developmental outcomes (MDI and PDI) in infants with and without ROP. In univariate analysis infants with any ROP had significantly lower TBV, UWMV, cerebellar volumes, MDI, and PDI than infants without ROP.

Multiple linear regression analysis was performed in order to further evaluate the relationship between any ROP and brain volumes at term-equivalent age and neurodevelopmental outcome at 2 years of age. After adjustment for GA the relationships between any ROP and TBV and GMV, respectively, did not remain significant and were not further evaluated in multivariate models. Figure 1a–c shows the relationships between any ROP and cerebellar volume, UWMV, MDI, and PDI, respectively.

Table 4 presents the contribution of any ROP to cerebellar volume, UWMV, MDI, and PDI following multivariate regression analysis. The relationships between any ROP and cerebellar and UWMV, respectively, re-

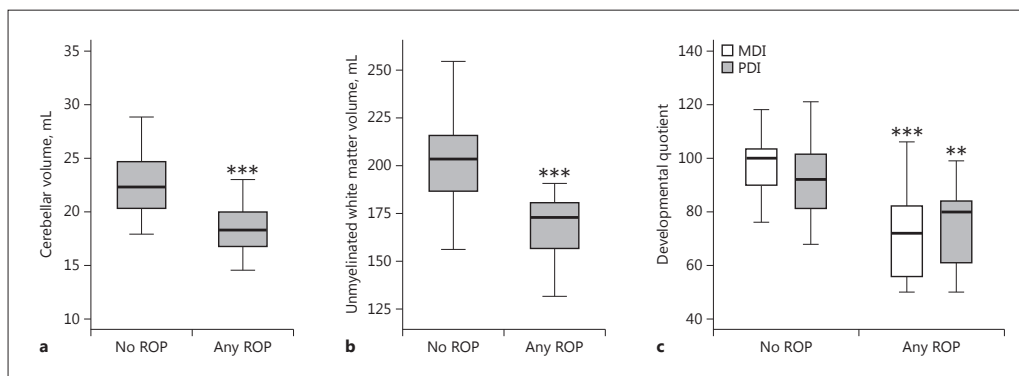


Fig. 1. Median and IQR of cerebellar volume (**a**), unmyelinated white matter volume (**b**), and Mental Developmental Index (MDI) and Psychomotor Developmental Index (PDI) (**c**) in relation to the presence or absence of retinopathy of prematurity (ROP). ** $p \leq 0.01$; *** $p \leq 0.001$.

Table 4. Contribution of any ROP to cerebellar volume and to UWMV at a postmenstrual age of 40 weeks and to MDI and PDI at 2 years of corrected age in a linear regression model

Outcome variable	<i>p</i> value	Any ROP (yes/no)	
		<i>R</i> ²	adjusted <i>R</i> ²
Cerebellar volume ^a	0.029	0.50	0.43
UWMV ^a	0.030	0.58	0.52
MDI ^b	0.007	0.38	0.29
PDI ^c	0.03	0.42	0.35

ROP, retinopathy of prematurity; UWMV, unmyelinated white matter volume; MDI, Mental Developmental Index; PDI, Psychomotor Developmental Index. ^a Included independent variables: any ROP (yes/no), gestational age (days), birth weight (g), gender, septicemia (yes/no), and total steroid intake (calculated hydrocortisone equivalents in mg/kg) from birth to a postmenstrual age of 35 weeks. ^b Included independent variables: any ROP (yes/no), gestational age (days), birth weight (g), gender, Apgar score <7 at 5 min, and total steroid intake (calculated hydrocortisone equivalents in mg/kg) from birth to a postmenstrual age of 35 weeks. ^c Included independent variables: any ROP (yes/no), gestational age (days), birth weight (g), gender, and severe brain damage (IVH grade III, and/or parenchymal hemorrhage, and/or WMD).

mained significant after adjustment for GA, BW, gender, septicemia, and total steroid intake. The relationship between any ROP and MDI remained significant after adjustment for GA, BW, gender, Apgar score <7 at 5 min,

and total steroid intake. The relationship between any ROP and PDI remained significant after adjustment for GA, BW, gender, and severe brain damage.

Discussion

Our main findings were that the presence of any stage of ROP was associated with a lower cerebellar volume and UWMV at term-equivalent age and with impaired cognitive and motor development at 2 years of corrected age. Thus, infants with less severe ROP (stage 1 or 2) exhibited a degree of brain volume reduction and neurodevelopmental impairment similar to that of infants with more severe ROP (stage 3).

Several studies have shown an association between ROP and impaired later developmental outcomes [2, 9–11]. The majority of studies compare severe ROP to no ROP and do not include less severe stages of ROP. A recent case-control study comparing severe ROP with no or stage 1 ROP found severe ROP to be associated with reduced cerebellar and brainstem volumes at term and with neurodevelopmental deficits at 15 months and 2 years of age [17]. One study considered different stages of ROP and found the severity of neonatal ROP to be a marker for functional disability at 5.5 years of age. They found high rates of disability in VLBW infants who develop severe ROP and subsequently have unfavorable visual outcomes [18].

Several features of retinal and brain development have links to common pathways that in turn may be affected by insults associated with very preterm birth. This is supported by similar risk factors being associated with ROP, lower brain volumes, and impaired neurodevelopment, which suggests common antecedent events that affect migration and proliferation in both the retina and the brain. The signaling and mitogenic protein and transcription factor sonic hedgehog is a key factor in both cerebellar and retinal proliferation during early development [19, 20].

In this study, ROP was primarily associated with a lower cerebellar volume and UWMV. These respective brain regions and the retina may present a common vulnerability due to depletion of the same trophic factors. IGF-1 is an anabolic hormone that is critical to both vascular and neural development. Low IGF-1 levels in preterm infants have been associated with ROP [21], decreased brain volumes, and impaired neurodevelopmental outcomes at 2 years of corrected age [7, 13].

The relationship between ROP and poor white matter development as determined by diffusion tensor imaging and tractography has been evaluated in one study where severe ROP predicted a white matter maturational delay in the optic radiations but also in other regions of posterior white matter. The delayed white matter maturation was present independently of other signs of brain injury [11]. The retinal nerve fiber layer as estimated by spectral-domain optical coherence tomography at term-equivalent age is thinner in very preterm infants and has been shown to correlate with white matter injury at term-equivalent age and later impaired neurodevelopment [22].

The presence of supratentorial brain injury has previously been associated with impaired cerebellar growth [23]. The presence of severe brain damage, as defined by cerebral ultrasound, was not related to any ROP or to the estimated cerebellar volume in the current study although the small number of infants with severe brain damage could have obscured such an association.

In addition to a lower cerebellar volume and UWMV, infants with any ROP had significantly lower MDI and PDI scores at 2 years of corrected age. Stage 1 ROP is sometimes difficult to diagnose and subtle forms may be missed at examination. However, similar findings were reported in another study, in which the stage of ROP *per se* did not determine the subsequent presence of neurodevelopmental impairment [9]. Several studies have also documented the importance of cerebellar volume, particularly with respect to cognitive function in preterm infants at 2 years of age [13, 24, 25].

The accumulated dose of steroid intake displayed a significant association with any ROP, lower cerebellar and UWMV, and lower MDI. Postnatal corticosteroid exposure in preterm infants has previously been related to an increased risk of ROP, an altered optic radiation structure, and impaired cerebellar growth [26–28]. The neonatal cerebellum has the highest number of glucocorticoid receptors [29]. In mice, glucocorticoids inhibit proliferation of cerebellar granular neuron precursors [30].

In conclusion, we found that the development of any stage of ROP in very preterm infants was associated with reduced brain volumes and impaired developmental outcomes at 2 years of corrected age. Though based on a small cohort of infants, our results suggest that any type of ROP, not just severe, should be considered in future studies on ROP and developmental outcomes. These findings also raise the possibility of common pathways essential for the development of the retina and brain. Therefore, strategies aimed at preventing any stage of ROP may also have neuroprotective effects on the developing brain.

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Disclosure Statement

Insulin-like growth factor 1 is covered by the patent owned by or licensed to Premature AB, Uppsala, Sweden. A.H., D.L., and I.H.P. own shares in the company with a financial interest in Premature AB. A.H., D.L., L.E.H.S., and I.H.P. work as consultants for Shire Pharmaceuticals (Lexington, MA, USA).

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References

- Laptook AR, O'Shea TM, Shankaran S, Bhaskar B, NICHD Neonatal Network: Adverse neurodevelopmental outcomes among extremely low birth weight infants with a normal head ultrasound: prevalence and antecedents. *Pediatrics* 2005;115:673–680.
- Hellgren KM, Tornqvist K, Jakobsson PG, Lundgren P, Carlsson B, Källén K, Serenius F, Hellström A, Holmström G: Ophthalmologic outcome of extremely preterm infants at 6.5 years of age: Extremely Preterm Infants in Sweden Study (EXPRESS). *JAMA Ophthalmol* 2016, DOI: 10.1001/2016.0391.
- Volpe JJ: Cerebellum of the premature infant: rapidly developing, vulnerable, clinically important. *J Child Neurol* 2009;24:1085–1104.
- Chen J, Smith LE: Retinopathy of prematurity. *Angiogenesis* 2007;10:133–140.
- Hellström A, Smith LE, Dammann O: Retinopathy of prematurity. *Lancet* 2013;26:1445–1457.
- Löfqvist C, Engström E, Sigurdsson J, Hård AL, Niklasson A, Ewald U, Holmström G, Smith LE, Hellström A: Postnatal head growth deficit among premature infants parallels retinopathy of prematurity and insulin-like growth factor-I deficit. *Pediatrics* 2006;117:1930–1938.
- Hansen-Pupp I, Hövel H, Hellström A, Hellström-Westas L, Löfqvist C, Larsson EM, Lazeyras F, Fellman V, Hüppi PS, Ley D: Postnatal decrease in circulating insulin-like growth factor-I and low brain volumes in very preterm infants. *J Clin Endocrinol Metab* 2011;96:1129–1135.
- Allred EN, Capone A Jr, Fraioli A, Dammann O, Droste P, Duker J, Gise R, Kuban K, Leviton A, O'Shea TM, Paneth N, Petersen R, Trese M, Stoessel K, Vanderveen D, Wallace DK, Weaver G: Retinopathy of prematurity and brain damage in the very preterm newborn. *J AAPOS* 2014;18:241–247.
- Beligere N, Perumalswamy V, Tandon M, Mittal A, Floora J, Vijayakumar B, Miller MT: Retinopathy of prematurity and neurodevelopmental disabilities in premature infants. *Semin Fetal Neonatal Med* 2015;20:346–353.
- Schmidt B, Davis PG, Asztalos EV, Solimano A, Roberts R: Association between severe retinopathy of prematurity and nonvisual disabilities at age 5 years. *JAMA* 2014;311:523–525.
- Glass TJA, Chau V, Gardiner J, Foong J, Vinnall J, Zwicker JG, Grunau RE, Synnes A, Poskitt KJ, Miller SP: Severe retinopathy of prematurity predicts delayed white matter maturation and poorer neurodevelopment. *Arch Dis Child Fetal Neonatal Ed* 2017;102:F532–F537.
- Hansen-Pupp I, Löfqvist C, Polberger S, Niklasson A, Fellman V, Hellström A, Ley D: Influence of insulin-like growth factor I and nutrition during phases of postnatal growth in very preterm infants. *Pediatr Res* 2011;69:448–453.
- Hansen-Pupp I, Hövel H, Löfqvist C, Hellström-Westas L, Cilio CM, Andersson S, Fellman V, Ley D: Circulatory insulin-like growth factor-I and brain volumes in relation to neurodevelopmental outcome in very preterm infants. *Pediatr Res* 2013;74:564–569.
- Marsal K, Persson PH, Larsen T, Lilja H, Selbing A, Sultan B: Intrauterine growth curves based on ultrasonically estimated foetal weights. *Acta Paediatr* 1996;85:843–848.
- International committee for the Classification of Retinopathy of Prematurity: The International Classification of Retinopathy of Prematurity revisited. *Arch Ophthalmol* 2005;123:991–999.
- Early Treatment for Retinopathy of Prematurity Cooperative Group: Revised indications for the treatment of retinopathy of prematurity: results of the early treatment for retinopathy of prematurity randomized trial. *Arch Ophthalmol* 2003;121:1684–1694.
- Drost FJ, Keunen K, Moeskops P, Claessens N, van Kalken F, Isgum I, Voskuil-Kerkhof E, Groenendaal F, de Vries LS, Benders MJ, Termote J: Severe retinopathy of prematurity is associated with reduced cerebellar and brainstem volumes at term and neurodevelopmental deficits at 2 years. *Pediatr Res* 2018, Epub ahead of print.
- Msall ME, Phelps DL, DiGaudio KM, Dobson V, Tung B, McClead RE, Quinn GE, Reynolds JD, Hardy RJ, Palmer EA: Severity of neonatal retinopathy of prematurity is predictive of neurodevelopmental functional outcome at age 5.5 years. Behalf of the Cryotherapy for Retinopathy of Prematurity Cooperative Group. *Pediatrics* 2000;106:998–1005.
- Haldipur P, Bharti U, Govindan S, Sarkar C, Iyengar S, Gressens P, Mani S: Expression of sonic hedgehog during cell proliferation in the human cerebellum. *Stem Cells Dev* 2012;21:1059–1068.
- Wang Y, Dakubo G, Thurig S, Mazerolle CJ, Wallace VA: Retinal ganglion cell-derived sonic hedgehog locally controls proliferation and the timing of RGC development in the embryonic mouse retina. *Development* 2005;132:5103–5113.
- Jensen AK, Ying GS, Huang J, Quinn GE, Binenbaum G: Postnatal serum insulin-like growth factor I and retinopathy of prematurity. *Retina* 2017;37:867–872.
- Rothman AL, Sevilla MB, Mangalesh S, Gustafsson KE, Edwards L, Cotten CM, Shimony JS, Pizoli CE, El-Dairi MA, Freedman SF, Toth CA: Thinner retinal nerve fiber layer in very preterm versus term infants and relationship to brain anatomy and neurodevelopment. *Am J Ophthalmol* 2015;160:1296–1308.
- Tam EW, Miller SP, Studholme C, Chau V, Glidden D, Poskitt KJ, Ferriero DM, Barkovich AJ: Differential effects of intraventricular hemorrhage and white matter injury on preterm cerebellar growth. *J Pediatr* 2011;158:366–371.
- Lind A, Parkkola R, Lehtonen L, Munck P, Maunu J, Lapinleimu H, Haataja L, PIPARI Study Group: Associations between regional brain volumes at term-equivalent age and development at 2 years of age in preterm children. *Pediatr Radiol* 2011;41:953–961.
- Van Kooij BJ, Benders MJ, Anbeek P, Van Haastert IC, de Vries LS, Groenendaal F: Cerebellar volume and proton magnetic resonance spectroscopy at term, and neurodevelopment at 2 years of age in preterm infants. *Dev Med Child Neurol* 2012;54:260–266.
- Movsas TZ, Spitzer A, Gewolb IH: Postnatal corticosteroids and risk of retinopathy of prematurity. *J AAPOS* 2016;20:348–352.
- Kelly CE, Cheong JL, Molloy C, Anderson PJ, Lee KJ, Burnett AC, Connelly A, Doyle LW, Thompson DK: Victorian Infant Collaborative Study Group: Neural correlates of impaired vision in adolescents born extremely preterm and/or extremely low birthweight. *PLoS One* 2014;9:e93188.
- Tam EW, Chau V, Ferriero DM, Barkovich AJ, Poskitt KJ, Studholme C, Fok ED, Grunau RE, Glidden DV, Miller SP: Preterm cerebellar growth impairment after postnatal exposure to glucocorticoids. *Sci Transl Med* 2011;3:105ra105.
- Pavlik A, Buresova M: The neonatal cerebellum: the highest level of glucocorticoid receptors in the brain. *Brain Res* 1984;314:13–20.
- Heine VM, Rowitch DH: Hedgehog signaling has a protective effect in glucocorticoid-induced mouse neonatal brain injury through an 11betaHSD2-dependent mechanism. *J Clin Invest* 2009;119:267–277.

