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THE ALTERATIONS OF THE CYTOKINE NETWORK IN PERINATAL HYPOXIC-ISCHEMIC BRAIN INJURY

PhD thesis

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1 ABBREVIATIONS

ACA	Anterior cerebral artery
ADC	Apparent diffusion coefficient
aEEG	Amplitude-integrated electroencephalogram
AIS	Acute ischemic stroke
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole receptors
APC	Antigen presenting cells
BBB	Blood-brain barrier
CD	Cluster of Differentiation
CNS	Central nervous system
CPISR	Canadian Pediatric Ischemic Stroke Registry
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen - 4
CSF	Cerebrospinal fluid
CSVT	cerebral sinus venous thrombosis
DW MRI	Diffusion-weighted magnetic resonance imaging
EEG	Electroencephalogram
EPO	Erythropoietin
FOXP3	Forkhead box protein 3
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GM-IVH	Germinal matrix - intraventricular hemorrhage
HEV	High endothelial venules
HI	Hypoxic-ischemic
HIE	Hypoxic ischemic encephalopathy
IDO	Indoleamine 2,3-dioxygenase
IFN-γ	Interferon - gamma
IL	Interleukin
IVH	Intraventricular hemorrhage
KYN	Kynurenine
KYNA	Kynurenic acid
LPS	Lipopolysaccharide
MCA	Middle cerebral artery
MCP-1	Monocyte chemoattractant protein - 1
MFI	Mean fluorescence intensity

MHC	Major histocompatibility complex
MIP-1β	Macrophage inflammatory protein - 1 beta
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
MSC	mesenchymal stem cell
NAIS	Neonatal arterial ischemic stroke
NE	Neonatal encephalopathy
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NOS	Nitric oxide synthase
PBMC	Peripheral blood mononuclear cells
PCA	Posterior cerebral artery
PET	Positron emission tomography
PVL	Periventricular leukomalacia
qPCR	Quantitative polymerase chain reaction
RAG1	Recombination activating gene 1
RNS	Reactive nitrogen species
RORγt	RAR-related orphan receptor gamma t
ROS	Reactive oxygen species
S1P	Sphingosine-1-phosphate
S1PR	Sphingosine-1-phosphate receptor
SEP	Somatosensory evoked potential
T-bet	T-box expressed in T cells
TCR	T cell receptor
TGF-β	Tumor growth factor - beta
Th1	Helper T lymphocyte -1
Th17	Helper T lymphocyte -17
Th2	Helper T lymphocyte -2
TNF-α	Tumor necrosis factor - alpha
Treg	Regulatory T lymphocyte
TRP	Tryptophan
VCAM-1	Vascular cell adhesion molecule - 1
VEP	Visual evoked potential
VLA-4	Very Late Antigen - 4
WT	Wild type

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2 INTRODUCTION

Perinatal asphyxia and the consequent hypoxic-ischemic brain injury continues to be a major cause of perinatal morbidity and mortality and lifelong disability, despite the advances of the past decades in prenatal diagnostic methods and neonatal intensive care. It is important to note, that the initial degree of injury and the clinical status of the neonate often do not allow accurate prediction of the eventual neurodevelopmental outcome. While some children show favorable outcome and no significant disability, others develop severe neurodevelopmental impairments such as mental retardation, sensory impairment, cerebral palsy and seizures. Identifying factors which could differentiate between mild and severe outcome would be of great clinical value.

It is becoming more and more clear, that the final outcome is influenced by several factors beyond the severity of the initial insult. One of the key factors determining the progress of brain injury is the neuroinflammatory response evoked by hypoxic-ischemic injury. Neuroinflammation is now recognized to be a common feature of many neurological disorders, including hypoxic-ischemic brain injury. However, it appears to have dual aspects. While a certain level of inflammatory response is part of the physiological recovery process of the central nervous system, excessive neuroinflammation has been shown to play an important role in facilitating further brain injury. Emerging evidence indicates, that neuroinflammation could continue for many months after the initial brain injury and this latent phase could play an important role in the long-term consequences of perinatal hypoxic-ischemic brain injury.

In the past decades important advances have been made in diagnosing perinatal hypoxic-ischemic brain injury. Introducing amplitude-integrated electroencephalogram monitoring into the routine clinical practice has enabled clinicians to follow the status of the neonate much more precisely. The widespread availability of MR imaging has opened new options for following the evolution of the brain injury and differentiating between asphyxia and disorders which are clinically overlapping, such as perinatal stroke. New MR modalities also allow earlier estimation of the injury with higher precision.

Inarguably one the most important advancements of the past decade has been the introduction of therapeutic hypothermia into the common clinical routine, which has been shown to improve neurological outcome significantly. One of the fundamental

effects of hypothermia is the amelioration of the neuroinflammatory response and consequent tissue injury. Current research is focusing on developing novel immunomodulatory agents, many of which primarily target the neuroinflammatory response.

The improving diagnostic possibilities in perinatal asphyxia also raised the awareness towards other etiologies of hypoxic-ischemic brain injury which are characteristic for the perinatal period. One of the most common is stroke, for which the neonatal period carries the highest risk in the entire childhood. Perinatal stroke is one of the most common causes of seizures, however it is seldomly diagnosed in a timely manner. The clinical presentation of the neonates often overlaps with global hypoxic-ischemic brain injury, and the real etiology is only later revealed upon MR examination. The pathophysiology of perinatal stroke is poorly understood; however it appears, that in-utero inflammation could be an important risk factor. Similarly to asphyxia, stroke also evokes a neuroinflammatory response, which plays an important role in the evolution of the brain injury. Disease specific preventive measures, prognostic factors and therapeutic strategies are not available, as neonates with stroke are not yet considered eligible for hypothermia therapy.

Although recent years' research has brought important aspects of the neuroinflammatory response to light, many questions remain. One area, where experimental data is especially scarce is the involvement of the adaptive immune system in neuroinflammation. Our research group has a longstanding interest in neonatal immune function and the role of human T cells in the pathophysiology of different diseases. T lymphocytes are key members of the adaptive immune system, exerting their effects largely via the cytokine network. In this study, we aimed to characterize the cytokine production of T lymphocytes. As previous studies have primarily focused on plasma cytokine levels, these data provide a novel insight into the function of T cells after perinatal hypoxic-ischemic brain injury. We also aimed to learn more about the latent phase of neuroinflammation to identify players of the inflammatory network, which could be responsible for this prolonged immune response. We therefore extended our study period to the entire first month of life.

By joining this research group in 2010, I have had the opportunity to rely on its long experience in the field of perinatal immunology and flow cytometry and carry out

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the measurements which provide the basis of this thesis. We hope that the results from our work could improve our understanding of the characteristics of this critical period of life and hopefully support the search for prognostic and therapeutic targets, which could open the opportunity for individualized care in the future.

2.1 THE NEONATAL BRAIN - SELECTIVE VULNERABILITY OR FUNCTIONAL PLASTICITY AFTER HYPOXIA-ISCHEMIA?

The human brain has a high requirement of oxygen and glucose, but lacks energy and nutrient reserves, and thus is highly dependent on a continuous blood flow (1). The hypoxic-ischemic (HI) injury of the developing brain is one of the leading causes of child mortality, morbidity and long-term disability (2). The developing fetal and neonatal brain responds very differently to HI injury than the mature adult brain. On the one hand, neonatal brain is able to withstand much longer periods of hypoxemia, than the adult brain (3). This is due to a number of adaptive mechanisms, such as the capacity to increase cerebral blood flow (4). On the other hand, severe hypoxia together with ischemia can initiate a self-sustaining cascade of neurotoxic events, which can last for weeks and result in delayed brain damage (5, 4, 6, 7). In the immature brain, the development of central motor pathways can be disturbed, and developmental plasticity can be altered (2). Certain cerebral regions of the developing brain are especially susceptible to HI injury, and these change as the fetus matures (8, 9). As our understanding of the characteristics of CNS development deepened, many circumstances have been revealed, which may be responsible for this increased vulnerability of the developing brain to hypoxia-ischemia (1). The most important factors determining the severity and type of perinatal HI injury are

1) The intensity and timing of HI injury in relation to birth
2) The degree of maturation of the CNS at the time of insult
3) The selective ischemic vulnerability
4) The characteristics of the subsequent neuroinflammatory response (1).

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Gestational age has a determinate impact on the characteristics of HI injury. For instance, preterm infants are much more resilient to hypoxemia than near-term infants. Premature fetal sheep can tolerate up to 25 minutes of complete cessation of blood flow, whereas in near-term fetuses the tolerable time of hypoxia is maximum 10 minutes (10, 11) This is most likely due to the gradual increase in the metabolic rate of the CNS as the fetus matures (8). Term neonates are also much more likely respond to severe HI injury with blood-brain barrier (BBB) breakdown, significant brain oedema and massive neuroinflammatory response causing the impairment of CNS metabolism, excitotoxicity, free radical production and high levels of apoptosis (8, 12-14).

Global hypoxia-ischemia most often damages specific structural groups (4, 15, 16). Gestational age and the level of CNS maturity determine the characteristics of the HI lesions and the associated clinical patterns of disability. The lack of cerebral circulatory autoregulation and the presence of fragile, poorly anastomosed blood vessels in the germinal matrix (17, 18) put preterm neonates at extremely high risk for germinal matrix - intraventricular hemorrhage (GM-IVH), one of the most devastating forms of permanent perinatal injury (Figure 1).

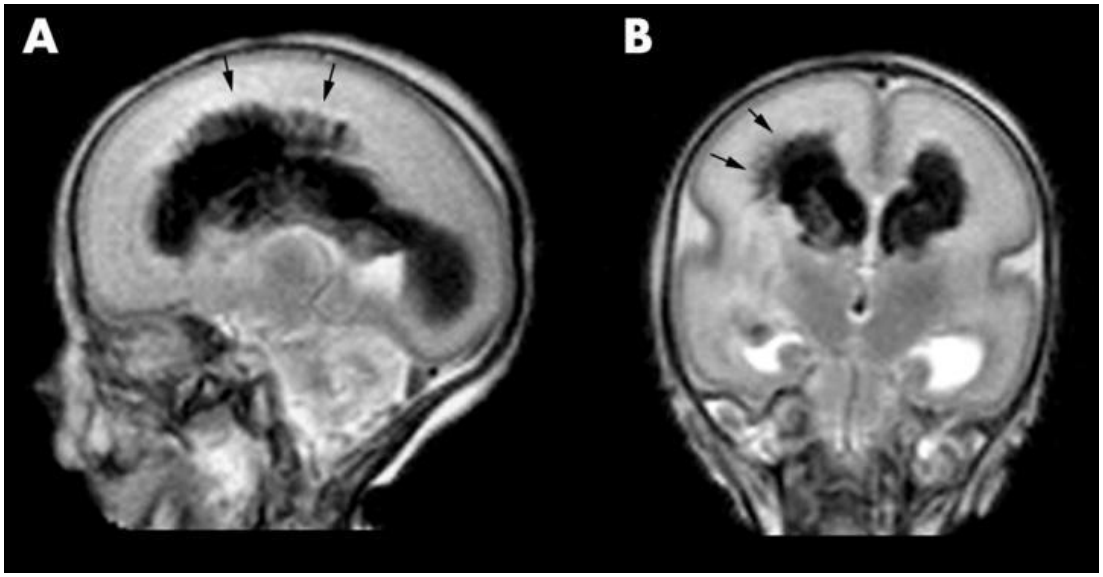

Figure 1. MRI image showing a characteristic injury of the premature brain, germinal matrix - intraventricular hemorrhage on both sides with parenchymal involvement on the right (arrows). *A) Sagittal and (B) coronal T2 weighted FSE image of an infant at 27 weeks GA. The images were published by Counsell et al. (19).*

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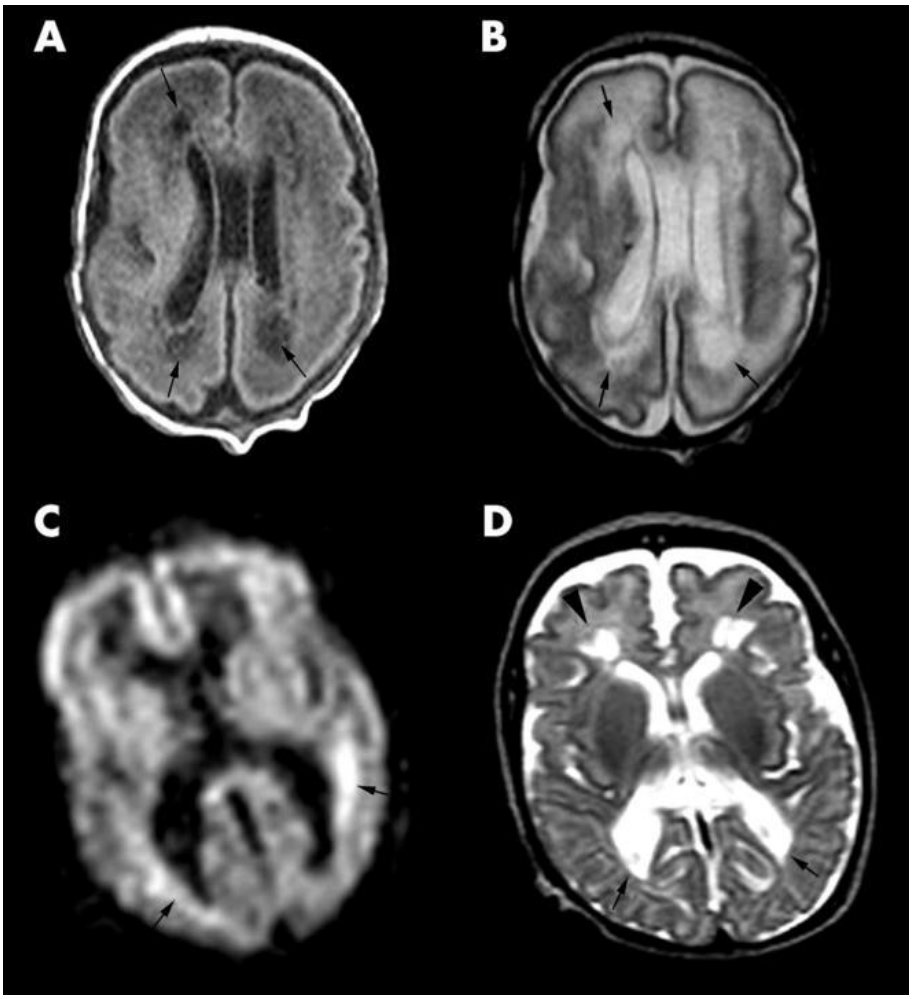

Figure 2. MRI image showing a characteristic form of injury of the premature brain, periventricular leukomalacia (PVL). *Image was published by Counsell et al. (19), with the following figure legend: PVL in an infant at 28 weeks gestational age. (A) Transverse T1 weighted image at the mid-ventricular level showing cystic PVL as areas of low signal within the cerebral white matter posterior and anterior to the lateral ventricles (arrows). (B) Transverse T2 weighted FSE image at the mid-ventricular level showing the cystic lesions as high signal intensity (arrows). (C) DWI image showing areas of restricted diffusion around the lateral ventricles as high signal intensity (arrows). (D) T2 weighted FSE image of the same infant at 40 weeks GA showing squared off posterior horns of the lateral ventricles (arrows) and diminished white matter posteriorly. Cystic lesions are shown anterior to the anterior horns of the lateral ventricles (arrowheads)*

Until the 32nd week of fetal development, the selective vulnerability of late oligodendrocyte progenitors (20) make the periventricular white matter especially susceptible to damage, making periventricular leukomalacia (PVL) (Figure 2), often associated with spastic palsy, the other common form of injury in preterm neonates (4). The consequent motor, sensory and cognitive deficits show specific patterns characteristic for preterm brain injury, for example cortical visual impairment (2).

Term neonates on the other hand, respond with different patterns of injury to severe or "near-total asphyxia". Based on magnetic resonance imaging (MRI), the most common types of damage are parasagittal cortical injury and the symmetric injury of basal ganglia, the thalamus, hippocampus and the peri-Rolandic cerebral cortex (21-26), often along with brain stem injury (Figure 3). The fragility of vessels decreases by this time and if IVH is present, it usually is associated to thalamic bleeding (13). Periventricular white matter damage is also uncommon, although transient MRI-signal alterations often show the involvement of the posterior internal capsule (25, 27). The associated clinical symptoms in term neonates are severe, permanent motor impairment with rigidity, which affects the upper extremities more than the lower ones and motor speech impairment (22, 4, 24). One factor, which can influence the gestation-age dependent pattern of HI injury is the localization of the "watershed" boundary zones, which changes as the vascularization of the fetal brain evolves (8). In term infants these lie between the major cerebral arteries and overlap with the structures most affected by HI. Although cerebrovascular factors might have an impact on the initial HI insult, it appears, that the endogenous vulnerability of certain structures in the developing brain might play a much more important role in determining the final pattern of injury and disability (4).

The selective vulnerability of the developing brain to hypoxic-ischemic injury has been the focus of intensive research for decades, however, many questions remain. One working model proposes, that the key to understanding selective neuronal vulnerability in term infants lies in the position of the vulnerable structures within the maturing excitatory neuronal circuits (28). It suggests, that excitotoxicity, which is the selective death of neurons and glia due to the overstimulation of excitatory neurotransmitter - mainly glutamate – receptors, is one of the key processes in the pathological development of HIE. In fact, every vulnerable region in term neonates

(the thalamus, putamen and peri-Rolandic cortex) receives major excitatory glutamatergic input (29).

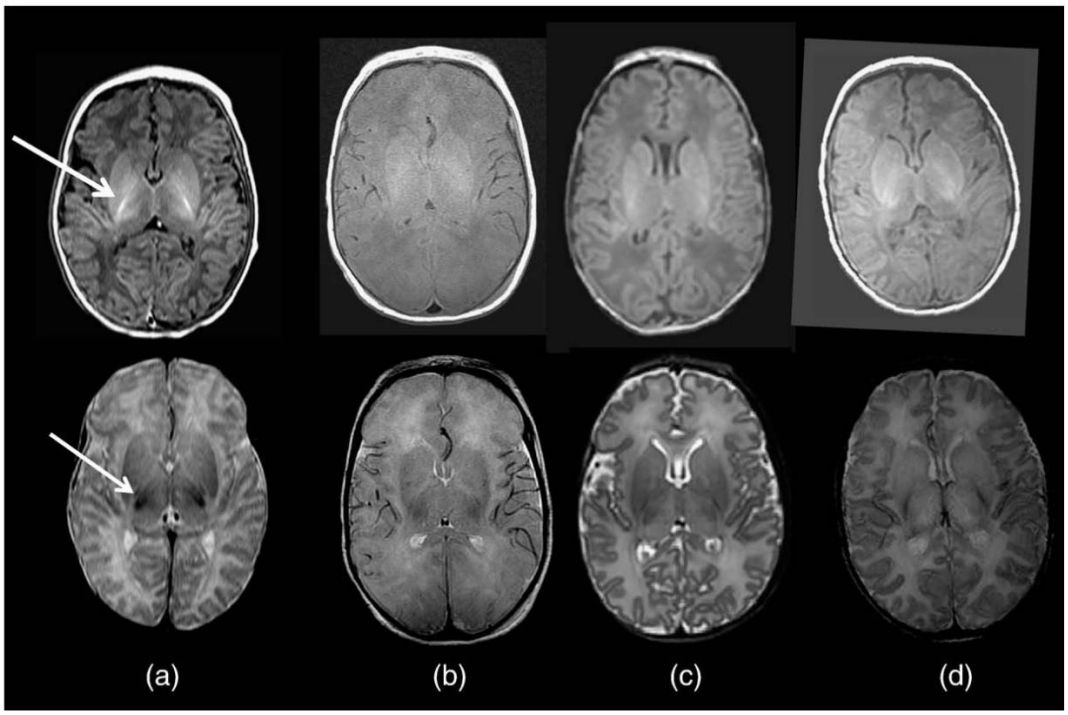

Figure 3. MRI images comparing the brain of a healthy term neonate with three term neonates with HIE. *This image was published by Rutherford et al. (30) with the following legend: the posterior limb of the internal capsule. T1 weighted images (top row) with T2 weighted images (bottom row). (a) Normal appearances of the posterior limb of the internal capsule (arrows). (b, c, d) Abnormal appearances of paired T1 and T2 weighted sequences in three different infants with HIE.*

Authors hypothesize, that these circuits become hyperactivated and the synapses are flooded with glutamate, causing excitotoxicity, which triggers selective neuronal death (4, 31, 32, 6). This model also explains why structures such as the internal and external segments of the globus pallidus are spared during HIE, but not in other disorders, such as kernicterus (28), since they are strongly inhibited during the hyperactivation of these circuits (22). Furthermore, the inhibition of the globus pallidus, alleviates the inhibition of the thalamus, which leads to enhanced cortical stimulation. This is consistent with the clinical signs of HIE, which indicate cortical hyperactivity, such as seizures and abnormal electroencephalographic

(EEG) background activity (3, 6, 33). Further evidence suggests, that vulnerable regions show selective glucose hypermetabolism (6), which appears to reflect enhanced glutamate release and reuptake at the hyperactive excitatory synapses (34) and correlate with outcome (35).

Oxidative stress appears to be the other major cause of neuronal damage following perinatal HI injury by triggering apoptosis via the mitochondria along other effects (36). Oxidative stress emerges due to the imbalance between the production and neutralization of free radicals, such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) (8). The developing brain is highly vulnerable to oxidative stress, much more than the adult brain due to its higher lipid content (37), the uniquely high levels of polyunsaturated fatty acids, its high oxygen requirement and thus, increased ROS-generating capacity, the low concentrations and activity of antioxidants and the higher levels of redox-active iron (38-43).

Nitric oxide (NO), although an important physiological mediator (44), when produced in excess amounts, has neurotoxic effects via the generation of RNS, such as peroxynitrite (OONO) (45). RNS-mediated damage includes lipid peroxidation, impairment of mitochondrial respiration and the induction of ROS production (46-48). The nitric oxide synthase (NOS) enzyme has three major isoforms: the neuronal NOS (nNOS), the endothelial NOS (eNOS), and the inducible NOS (iNOS), which is mainly found in activated inflammatory cells (8, 49). Increased NO production in specific cerebral regions appears to be an important component of selective ischemic vulnerability (50). Cells expressing the immature form of *N*-methyl-D-aspartate (NMDA) glutamate receptor are generally highly resistant to HI injury and NMDA mediated excitotoxicity (51, 52) and are abundant in regions, which are known to be selectively vulnerable to HI, such as the basal ganglia and thalamus (53). Increased stimulation after HI injury via this NMDA receptor leads to robust NO production due to the up-regulation of nNOS (54). Both the elimination of these neurons and the inhibition of NMDA coupling lead to the amelioration of ischemic injury (55). Furthermore, nNOS knockout in mice proved to be highly neuroprotective (56, 57). The inhibition of nNOS and iNOS also improved long-term functional outcome (58). Overall, it appears, that excitotoxicity and oxidative stress are the two most important mediators of perinatal HI brain damage and are key to understanding

selective ischemic vulnerability (8, 50).

Another important characteristic of the immature brain is, that neurons are much more prone to undergo apoptosis than in the adult brain, as programmed cell death is an important mechanism for refining newly formed neuronal pathways in the developing brain (59, 50, 60, 61). Compared with adult acute ischemic stroke (AIS), the ratio of apoptotic vs necrotic cells is much higher in the basal ganglia and cerebral cortex of term neonates one week after HI injury (62, 4, 63). This suggests, that apoptosis is a key step in the pathomechanism of HI brain injury, as is indicated by the elevated level of death-receptor proteins in the brain and cerebrospinal fluid (CSF) of neonates after HI brain injury (64, 65).

There is clear evidence from imaging studies and animal models, that the HI injury in the neonatal brain evolves in the first days to weeks after the initial insult (66) and the majority of the cell loss happens as a consequence of the ensuing neuroimmune response (67-69). The release of excitatory and inflammatory signals promotes robust microglial activation, initiating leukocyte migration to the CNS, which in return induce further inflammation. This cascade of events known as neuroinflammation, leads to the release of a wide range of inflammatory mediators, increased excitotoxicity and oxidative stress and the further destruction of the BBB (8). These processes all lead to accelerated cell death in the immature brain via apoptosis or necrosis (50). The growing understanding of the selective ischemic vulnerability of the developing brain has also opened the chance for new immunomodulatory therapeutic approaches in the form of NMDA antagonists combined with caspase inhibitors, which could yield an enhanced neuroprotective effect by inhibiting excitotoxicity and apoptosis at the same time (4, 70, 71).

Along the increased vulnerability, it is also important to consider, that the neonatal brain has a remarkable ability to recover and remodel after HI injury, due to its high level of neuroplasticity. Synaptic organization and the development of white matter pathways continues for years after birth (72, 73). One of the most relevant of these processes, is the activity-dependent plasticity of the descending motor pathways, which can influence outcome after unilateral hypoxic brain injury (74). Therefore, there is intensive research regarding neurodevelopmental techniques, which could alter this process in a favorable manner (74). Several animal models have confirmed,

that normal corticospinal tract connectivity and motor cortical map can be re-established, and motor function can be improved (75, 76) with electrical stimulation and/or targeted physiotherapeutic training.

It appears, that the neurogenesis capacity of the developing brain is not damaged by HI, in fact, in vivo data shows, that neural stem cell proliferation increases in the subventricular zone after HI in mice (77, 78). Evidence also indicates, that compared to the mature, adult brain, where remodeling is very limited, the developing brain has great capacity to modify its structure and function to overcome functional loss after HI injury (79, 80, 73). The developing brain retains its ability to "recruit new areas and form new circuits by reorganizing the neural network" (81, 82). Jung et al. utilized novel MR imaging techniques to demonstrate neuroplasticity on many levels, from cellular to systemic changes, such as cortical remapping during the spontaneous functional recovery of mice who sustained neonatal HI injury. They found, that the contralesional hemisphere was able to take over the governing of the motor function of both sides, which authors interpret as "spontaneously generated active plasticity with brain laterality established autonomously via the modification of neural circuitry". This process was associated with the recovery of motor functions (73). However, it is important to distinguish "good plasticity" from "bad plasticity". As an example, excessive stimulation of developing neuronal circuits due to HI injury and subsequent seizures, can lead to altered signaling and neurotransmission, wrong targeted innervation and the interruption of developmental apoptosis. This could result in abnormal neural circuitry, which contribute to epilepsy, motor and cognitive impairment (83, 84).

In conclusion, although, a growing body of evidence supports the higher neuroplasticity of the developing brain compared to the adult brain, it still appears, that the immature brain sustains far worse neurodevelopmental outcomes following severe ischemic injury (2). This indicates, that the crucial vulnerabilities of the developing brain outweigh its enhanced capacity for regeneration (83).

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2.2 PERINATAL ASPHYXIA

2.2.1 DEFINITION AND INCIDENCE

Several terms are often used as synonyms to describe the clinical syndrome, which evolves in the days following HI injury in neonates such as hypoxic-ischemic encephalopathy (HIE), neonatal encephalopathy (NE), perinatal asphyxia and birth asphyxia (85). As per the definition of Nelson and Leviton, NE is a "clinically defined syndrome of disturbed neurologic function in the earliest days of life in the term infant, manifested by difficulty with initiating and maintaining respiration, depression of tone and reflexes, subnormal level of consciousness, and often by seizures". This definition does not however specify etiological cause, and in fact, not all neonates who present with NE have an obvious history of perinatal hypoxic-ischemic episode (86). According to the definition of Volpe, hypoxemia is the "diminished amount of oxygen in the blood supply", whereas cerebral ischemia is the "diminished amount of blood perfusing the brain". As detailed previously, evidence shows, that the latter has greater impact in neonates, as glucose is also deprived from the developing brain along with oxygen, contributing to the primary energy failure (85, 87). The term asphyxia in addition refers to the "impairment of the exchange of blood gases oxygen and carbon dioxide", which occurs in the fetus if the umbilical or placental perfusion is compromised (85). To try to maintain a temporary homeostasis, the fetal circulation becomes rearranged, reducing nonobligatory energy consumption and increasing the blood flow of the brain, heart and adrenal glands. However, glucose reserves are rapidly consumed, leading to anaerobic metabolism and severe metabolic acidosis, due to lactate accumulation (2). In the clinical practice, indirect markers are used to estimate the degree of hypoxia-ischemia, such as low Apgar scores, decreased peripheral blood pH and acid-base parameters indicating metabolic acidosis. If these alterations are present along with the signs of encephalopathy (altered state of consciousness, hypotonia, abnormal reflexes, clinical seizures (88)), the diagnosis of hypoxic-ischemic encephalopathy can be established (85). Although there is a significant overlap in the above definitions, for the sake of clarity, the terms perinatal asphyxia and consequent hypoxic-ischemic encephalopathy (HIE) will be used in this thesis.

The incidence of HIE ranges between 1.0 and 8.0 per 1000 live births worldwide, with higher percentages in the developing world (85). In the developed world, the incidence of neonatal encephalopathy (NE) is between 2.5 and 3.5 per 1000 live births with a combined point estimate of 3.0 per 1000 live births, while the incidence of HIE is estimated to be between 1.3 and 1.7 per 1000 live births with a combined point estimate of 1.5 per 1000 live births. (85).

Perinatal asphyxia continues to be a major cause of perinatal mortality and long-term disability, despite the advances in neonatal healthcare and the introduction of therapeutic hypothermia as part of standard care (2). Perinatal asphyxia is accountable for approximately one million (23% of all) neonatal deaths worldwide (89). In the developed world, at least 0.5% of term neonates required resuscitation after birth due to a 5-min Apgar score of 6 or less (90), and in 2013 in the Netherlands at least 0.2% of all term neonates were severely depressed with a 5-min Apgar score of 3 or less. Among the latter group mortality was 24% (91).

Even though some surviving children show favorable neurological outcome, many sustain neurodevelopmental disabilities ranging from fine motor impairments and developmental delay to severe hindrances such as mental retardation, sensory impairment, cerebral palsy and epilepsy (92). Data indicates, that despite the significant reduction of perinatal morbidity in the developed world, the incidence of cerebral palsy has remained almost the same in the past 40 years (8, 93). The degree of individual variability in neurological outcome after perinatal asphyxia is especially intriguing, since it shows little correlation with the initial clinical status. It is thus crucial to constantly improve our understanding of the pathophysiology of HIE, since the key pathological factors regarding outcome are likely to be part of the neuroimmune response of the neonate to the initial HI insult.

2.2.2 RISK FACTORS

Several studies aimed to identify the potential risk factors of HIE, however, there was a high level of variability between the studies. In an Italian case-controlled study, out of the included 30,580 infants, 27 (0.09%) developed NE. In 26% of the cases only antepartum risk factors were present, in 22% only intrapartum risk factors could be identified, including acute intrapartum events, whereas in 44% of the cases both antenatal and peripartum risk factors were present. In 2 cases (7%) however, no risk factors could be identified (94). In the largest case-controlled study performed in Western Australia, in 69% of cases only antepartum risk factors were present, whereas only intrapartum risk factors could be identified in just 4% of the cases. In 22% of cases both were present, and in 2% no risk factors could be identified. There is also much discussion regarding the timing of the HI insult, however, prospective MRI based studies indicate, that the majority of HI injury is likely to occur at or close to the time of birth (95).

Several risk factors of NE have been identified, many of which are common in developed and developing countries. Identifying modifiable risk factors is a key point in current research. Risk factors, which have been shown to be associated with perinatal HIE are shown in Table 1. The presented information has been collected from the two largest case-controlled studies, one from Western Australia and the other from Nepal. Interestingly, there is a considerable overlap in the risk factors, despite the major differences in the socioeconomic background of the patients in the two studies.

Antepartum maternal risk factors include older maternal age, hypertension, thyroid disease, infertility treatments, severe pre-eclampsia and several sociodemographic factors (employment, insurance, etc.). Intrapartum complications such as cord abnormalities, maternal fever or prolonged membrane rupture to delivery interval resulted in a non-significant increase in the risk of NE in the Western Australian study. Non-cephalic presentation, forceps delivery, emergency section and catastrophic intrapartum events such as placental abruption or uterine rupture were associated with HIE (96, 97, 50, 85).

Of the fetal risk factors the most common were growth restriction in Western Australia and twin pregnancy in Nepal. Risk factors also included fetal distress and meconium stained amniotic fluid, however these are also the consequences of

intrauterine hypoxia and thus cannot be considered independent risk factors (96, 97, 85). Postnatal complications were present in less than 10% of cases and were severe respiratory distress, shock and sepsis (50).

Table 1. Summary of the risk factors of neonatal encephalopathy (NE). *The data were collected from the two largest case-control studies. [1] significant risk factor in Western Australian study, [2] significant risk factor in Nepal study (98, 96, 97).*

Maternal risk factors	Fetal risk factors
Antepartum	
Higher maternal age[1,2]	Gestational age > 41 weeks[1]
Primiparity[2]	Intrauterine growth restriction[1]
Family history of seizures and neurological disorders[1]	Abnormal placenta[1]
	Twin pregnancy[2]
Short stature (<145 cm)[2]	
Infertility treatment[1]	
Hypertension[1]	
Thyroid disease[1,2]	
Severe anemia[2]	
Vaginal bleeding[1]	
Viral infection[1]	
Severe preeclampsia[1]	
Lack of antenatal care[1,2]	
Sociodemographic factors[1]	
Intrapartum	
Pyrexia[1]	Non-cephalic presentation[2]
Prolonged interval from rupture of membranes to delivery[1,2]	Persistent occipitoposterior presentation[1]
Abnormalities in blood pressure[1]	Nuchal cord[1], cord prolapse[1,2]
Instrumental delivery[1,2]	Shoulder dystocia[1]
Emergency section[1,2]	Fetal distress[1]
Induced delivery[2]	Meconium stained amniotic fluid[1,2]
Acute intrapartum event[1,2]	

2.2.3 PATHOPHYSIOLOGY

Perinatal asphyxia evokes the injury of the central nervous system (CNS) due to the severe lack of oxygen and perfusion, leading to hypoxic-ischemic brain injury. In the initial phase, due to circulatory redistribution, a temporary compensation can be maintained, where nonobligatory energy consumption is minimized, cerebral blood flow is increased regionally, and neuronal activity is suppressed (99, 100, 2, 101). After a while cerebral blood flow gradually decreases due to either regional vasoconstriction or the progressive collapse of cardiac output (8, 102, 100). This leads to reduced oxygen delivery, decreased oxidative phosphorylation causing the depletion of high energy phosphate compounds (i.e. ATP), the rapid consumption of glucose reserves, and severe lactate acidosis, as a result of anaerobic metabolism (103-105, 2). This primary energy failure damages the BBB, leads to cerebral edema and initiates excitotoxicity and oxidative stress, culminating in the first wave of necrotic and apoptotic cell death. (8, 106, 107, 14).

If neonatal resuscitation is successful, glucose and ATP reserves are temporarily restored. However, this recovery phase is followed by the second wave of energy failure within 6 hours, as the HI brain injury continues to develop over the next weeks (2, 108, 6). The main features of the second phase are the secondary energy failure, characterized by an even more pronounced exhaustion of cellular ATP stores, further increase in lactate levels and pH alterations (109, 110, 2), the robust neuroinflammatory response and the decrease of cerebral autoregulation (8, 111, 112, 40). ATP deprivation combined with lactate acidosis leads to the failure of active membrane transport, leading to intracellular accumulation of Na^+, Ca^{2+}, and Cl^- ions. Consequently, prolonged neuronal membrane depolarization occurs, leading to excessive presynaptic glutamate release (113). In the absence of ATP and glucose, the function of glutamate reuptake pumps on perisynaptic glial cells is compromised (4, 34), leading to the accumulation of extracellular glutamate and other excitatory neurotransmitters, which causes the overstimulation of ionotropic glutamate receptors such as NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazole (AMPA) receptors (40). This leads to excessive calcium influx, initiating the excito-oxidative cascade. Intracellular calcium overload activates lipases, proteases and endonucleases, leading to the degradation of the cytoskeleton, mitochondria dysfunction, the swelling of the

cell and eventually apoptosis or necrosis (8, 4, 114, 2). Membrane depolarization and excessive excitatory stimulation can also induce seizures (115). Since glutamate receptors are vital regulators of the fetal brain development, they are expressed abundantly, therefore severe fluctuations in excitatory input have massive detrimental consequences (116, 40). The neuroinflammatory response is interconnected with this vicious circle in many ways. The activation and invasion of inflammatory cells results in the robust release of cytokines and other mediators, which continue to exacerbate the excito-oxidative cascade (8, 40) (Figure 4). In light of the above, it is not very surprising, that the majority of accelerated cell death happens during this second phase, where most of the free radicals, inflammatory mediators and excitatory amino acids are released (67, 68, 2, 69).

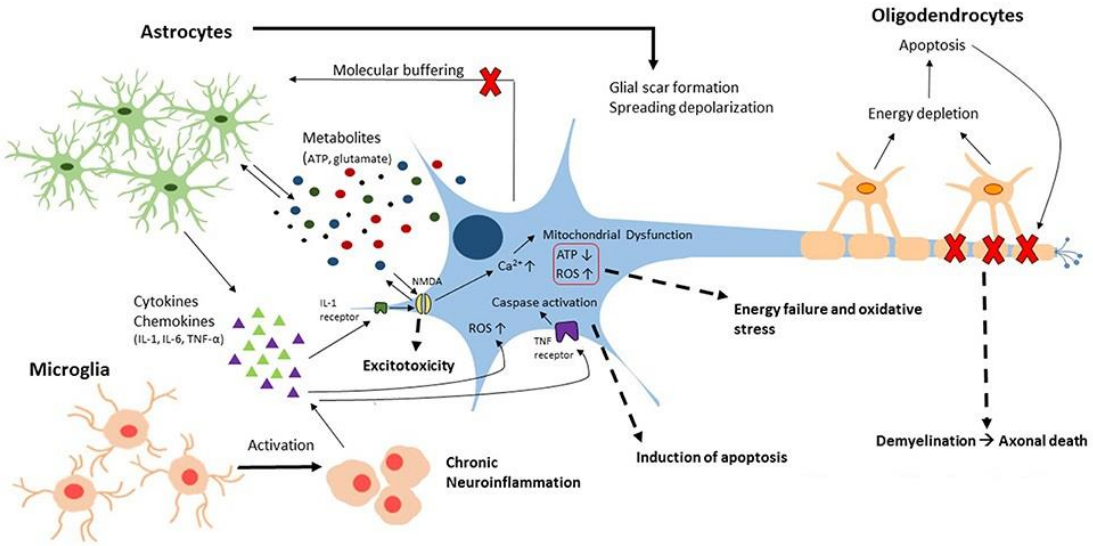

Figure 4. Key features of the neuroinflammatory response

This image was originally published by Sajja et al. in Frontiers (117) illustrating the contribution of glial cells to secondary brain injury.

Following the initial few days, the tertiary phase of the HI brain injury begins, which can continue for weeks after the initial insult. The primary mediators of this phase are the activated inflammatory cells and depending on the local homeostasis created by the neuroinflammatory response, the excito-oxidative injury can be sustained, and repair mechanisms can be stalled (118). The neuroinflammatory response is a complex process, which may have dual aspects being a hindrance, but

also a significant help in the recovery of the CNS. The substantial variability in outcome is likely related to the characteristics of the individual response to the initial HI insult, therefore understanding its characteristics is the primary aim of current research in the field. The neuroinflammatory response will be discussed in detail in Chapter 2.4.

2.2.4 CLINICAL PRESENTATION

Evidence indicates, that the majority of HI injuries happens during or in close proximity to birth (119). Term neonates generally present with symptoms of perinatal asphyxia directly after birth. The first mode of assessment is the Apgar score (120, 91), which is an evaluation of the cardiac, pulmonary and neurological status of the neonate at 1, 5 and 10 minutes after birth. The consequences of a severe HI insult are often already present at birth, indicated by a 5-min Apgar score under 5. Further signs of severe asphyxia include acidosis within 60 minutes of birth indicated by "umbilical-cord, arterial, or capillary pH of <7.00 or base deficit of ≥16 mmol per liter, signs of moderate-to-severe encephalopathy (lethargy, stupor, or coma), abnormal tone (focal or general hypotonia or flaccidity), abnormal reflexes (absent or weak suck or Moro response, sometimes oculomotor or pupillary abnormalities) and clinical seizures" (121).

Symptoms usually evolve during the first days of life and their severity and persistence is in correlation with the severity and duration of the HI insult (50, 91, 122, 6). Based on the clinical presentation, HIE can be classified as mild, moderate or severe (Table 2). Mild HI insult might manifest in subtle neurological abnormalities (such as transient drowsiness) without multi-organ damage, whereas a severe insult usually leads to moderate-to-severe HIE and variable degree of multi-organ failure (91, 123).

During the first 12-24 hours neonates with mild HIE generally show some neurological alterations, such as restlessness, moderate hypertonia, often associated with periodic breathing and feeding difficulty, which are likely associated with "abnormal stimulation or transient malfunction of the brain" (91). The most common systemic symptom is decreased urinary output. After 24 hours neonates with mild-to-moderate HIE can improve in their overall clinical and neurological status. They often

reach normal level of consciousness and begin to tolerate feeds during the first week of life. Laboratory parameters usually show a similar tendency (91).

Neonates with moderate-to-severe HIE on the other hand often show more profound neurological alterations during the first days, such as “depressed state of consciousness, gazing with abnormal papillary size, hypotonia, periodic breathing with apnea, bradycardia, and signs of seizure activity” (50, 91). In the most severe cases, neonates are often lethargic, and stupor and early onset, overt seizure activity is also common (91). In term infants, seizures are usually multifocal and clonic and can manifest in apneic spells (91). Systemic symptoms almost always include kidney damage and respiratory insufficiency with prolonged apnea, which often makes mechanic ventilation necessary around 48-72 h of life. HI injury can cause cardiomyopathy, with the need for inotropes, the severity of which is in close relations with the severity of the HI insult (124). As the HIE progresses, after 24 hours either a gradual stabilization or deterioration can be observed. In the first case, a gradual stabilization of physical parameters and improvement of alertness level occurs, however, neurological abnormalities, such as “hypo- or hypertonia (in the case of basal ganglia damage), feeding difficulties, decreased suck, abnormal swallow” likely reside (91). Hypotonia and proximal limb, facial and bulbar muscle weakness with amplified reflexes can persist for months after HI brain injury (50). In the second case, a gradual deterioration occurs, most likely as a consequence of the secondary phase of HI injury and neuroinflammation (109, 68). Seizures often become refractory, indicated by “apneic episodes, shrill cry, and jitteriness” (50). Neonates’ consciousness level progressively deteriorates, as brain stem functions start to be affected, and mechanic ventilation is almost always inevitable. Responsiveness decreases, seizure activity increases, and pupils become dilated (91). In the most severe cases, neonates die within the first week of life. Autopsy-based studies revealed cytotoxic brain edema and extensive neuronal damage after lethal perinatal asphyxia (125, 91).

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2.2.5 DIAGNOSIS

2.2.5.1 CLINICAL STAGING

Prenatal diagnostic methods (such as fetal heart rate patterns, scalp blood gas, meconium passing) may indicate fetal distress, however, they are poor predictors of the development of HIE and of long term neurological outcome (91, 126). The Apgar score is a somewhat better tool for direct postnatal assessment, however it is still far from reliable. A 10 min Apgar score of 5 or less predicts perinatal HI with 43% sensitivity and 95% specificity (127). However, HI injury cannot be ruled out in neonates with a good Apgar score (91), as 1 min Apgar score of 3 or less was found in only 31% of neonates with HIE (128).

The clinical features of the first few days show better correlation with future neurodevelopmental outcome, therefore, the need for clinical staging became evident. The first clinical staging system of HIE was established by Sarnat and Sarnat in 1976, it defines 3 stages of HIE based on neurological status and EEG characteristics of affected neonates (91, 6). This scoring system was then modified by Fenichel in 1983, to define mild, moderate and severe HIE, by including only clinical features (129) (Table 2). The latter system became the standard mode of clinical staging of HIE in neonates in the past decades worldwide, however, neurodevelopmental outcome cannot be reliably predicted based on solely the clinical severity of HIE.

In the “TOBY (TOtal Body hYpothermia) - Whole body hypothermia for the treatment of perinatal asphyxial encephalopathy trial”, term neonates with moderate-to-severe perinatal asphyxia were selected for total-body moderate hypothermia, initiated within 6 hours of birth and continued for the first 72 hours of life (121). They established the inclusion criteria to identify clinical features indicative of a developing encephalopathy early on (88). A modified version of this criteria system is used in the clinical practice to select neonates eligible for therapeutic hypothermia (see below).

Table 2. Clinical staging of hypoxic-ischemic encephalopathy according to Sarnat and Sarnat (6) and Fenichel (129) *This table is based on the publication of Groenendaal et al. (91)*

	Stage 1	**Stage 2**	**Stage 3**
A) Sarnat and Sarnat			
Consciousness	Hyperalert	Lethargic or obtunded	Stuporous
Neuromuscular control	Normal	Mild hypotonia	Flaccid
Suck / Moro reflex	Normal	Weak	Absent
Oculo-vestibular / tonic neck reflex	Normal	Strong	Absent
Autonomic function	In general sympathetic	In general parasympathetic	Both depressed
Seizures	None	(Multi)focal	Decerebrated
EEG findings	Normal	Low voltage (δ/0 waves)	Isoelectric
Duration	< 24 h	2-14 days	Hours to weeks

	Mild	**Moderate**	**Severe**
B) Fenichel			
Consciousness	Irritable/hyperalert	Lethargic	Comatose
Tone	Mildly abnormal	Moderately abnormal	Severely abnormal
Suck reflex	Abnormal	Poor	Absent
Primitive reflexes	Exaggerated	Depressed	Absent
Seizures	Absent	Present	Present
Brain stem reflexes	Normal	Normal	Impaired
Respiration	Tachypneic	Occasional apnea	Severe apnea

The following criteria are used to select neonates eligible for therapeutic hypothermia. Neonates meeting both criteria A and B may be considered for treatment with cooling.

A) Infants ≥36 completed weeks gestation admitted to the neonatal unit with <u>at least one of the following</u>:
Apgar score of ≤ 5 at 10 minutes after birth
Continued need for resuscitation, including endotracheal or mask ventilation, at 10 minutes after birth
Acidosis within 60 minutes of birth (defined as any occurrence of umbilical cord, arterial or capillary pH < 7.00)
Base Deficit ≥ 16 mmol/L in umbilical cord or any blood sample (arterial, venous or capillary) within 60 minutes of birth
B) Seizures or moderate to severe encephalopathy, consisting of:
Altered state of consciousness (reduced response to stimulation or absent response to stimulation) AND
Abnormal tone (focal or general hypotonia, or flaccid) AND
Abnormal primitive reflexes (weak or absent suck or Moro response)

2.2.5.2 NEUROPHYSIOLOGY

The assessment of neonates' brain activity by EEG has become an important tool in clinical staging, evaluating eligibility for therapies such as hypothermia, and predicting outcome (130, 131, 91, 132-134). Since the characteristics of the background activity in the first few days of life appear to be more important than those of the seizure activity, conventional multi-channel EEG recordings have limited value, as they give a detailed overview of a short time period (30-45 minutes) (135). In clinical practice, the usage of a simplified method, the amplitude-integrated EEG (aEEG) has become widespread, as it is easy to apply, correlates well with conventional EEG data and allows a continuous monitoring of brain activity in the first days of life (91, 136). Data from aEEG monitoring has predictive value, especially of severe injury, as suppressed background activity after 24 hours, or flat or continuous low voltage in the first 6 hours was almost always associated with poor outcome (137, 91, 138, 139). However, in neonates, where the background activity becomes normal within 24 hours, 39% still developed some neurological disability later on (139). On the other hand, continuous normal background activity was associated with normal

outcome (91, 138). Evoked potentials (EP) are electrical responses to repetitive visual, auditory or somatosensory stimulation, which can be assessed on the EEG. Visual and somatosensory evoked potentials in the first week of life (VEP and SEP respectively) also correlate with outcome, however, their clinical use is much less common than that of the aEEG (137, 91).

2.2.5.3 NEUROIMAGING

Although cranial ultrasound has been used for many years and is a good tool for bedside monitoring, its sensitivity after perinatal HI injury is far exceeded by magnetic resonance techniques (91). In the past decades, cranial magnetic resonance imaging (MRI) has become the standard diagnostic method following perinatal HI injury (95, 140) (Figure 5). MRI is the most informative, when performed between 4-7 days of life, as HI brain lesions acquired in the perinatal period are the most likely to be visible at this time period (141, 91). Following perinatal HI, the classical pattern of injury on MRI images is abnormalities in the thalamus and basal ganglia, and the surrounding white matter in the most severe cases (91, 142). Specific modalities, such as diffusion-weighted (DW) MRI are highly sensitive to early changes elicited by HI injury, especially if cranial mapping is performed by calculating the apparent diffusion coefficient (ADC) of water, based on which early cytotoxic edema can be differentiated from vasogenic edema (91, 143). Although DWI is a sensitive mode of evaluating the ischemic injury in the early phase, it often underestimates the final extent of damage, especially in the basal ganglia and thalamus (30).

Cranial magnetic resonance spectroscopy (MRS) can also be a valuable tool, as it provides insight into the metabolic changes of the CNS following perinatal HI. Poor neurological outcome was associated with elevated lactate level in the basal ganglia and thalamus of neonates on ^{1}H-MRS (144). Lower levels of phosphocreatine and ATP also correlated with worse outcome (91, 109, 68).

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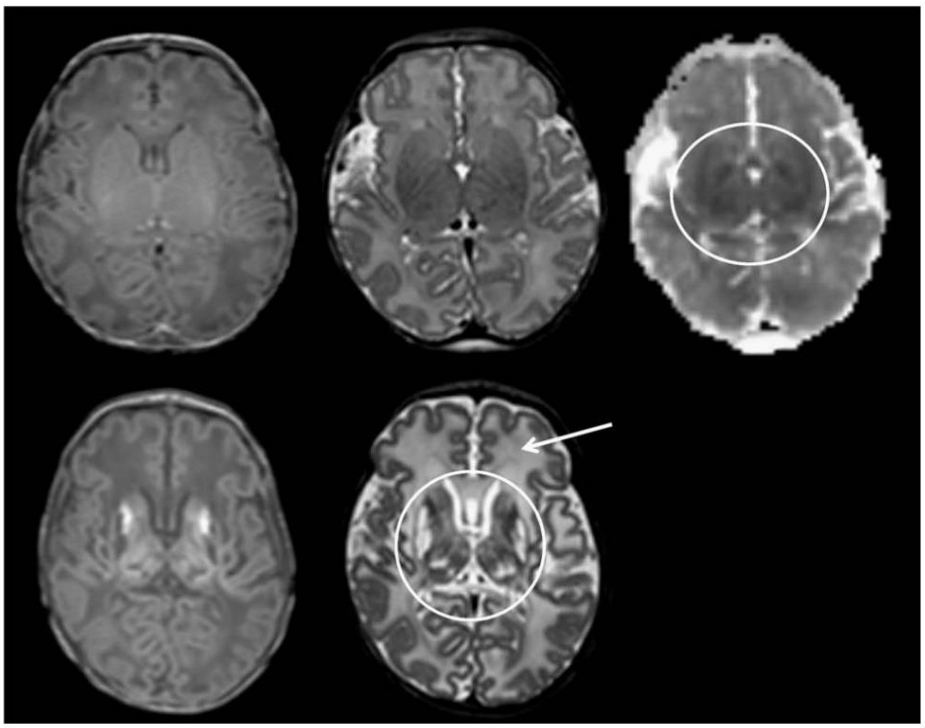

Figure 5. Different MRI modalities for assessing perinatal HIE. *This image was published by Rutherford et al. (30) with the following legend: Diffusion-weighted imaging in the basal ganglia and thalami. Top row. T1 (left) and T2 (middle) weighted images and ADC map (right) acquired on day 3. The ADC map shows several regions of abnormal low signal intensity within the basal ganglia and thalami (BGT) that look relatively subtle (circular region of interest). Bottom row. Day 22. T1 (left) and T2 (right) weighted images. The BGT abnormalities are very severe with no normal looking tissue within the BGT (circular region of interest). Any abnormality within the BGT on an early ADC map is likely to be clinically significant and any one diffusion-weighted examination is likely to underestimate the final lesion load. By day 22 the white matter signal intensity (arrow) has become abnormally high and there has been no head growth in this neonate.*

2.2.6 THERAPY

Neonates born with poor Apgar score due to perinatal asphyxia often require perinatal resuscitation and subsequent intensive supportive care in the first weeks of life. The most important aspects of supportive care are adequate ventilation and oxygenation, correction of acid-base status, cardiac support, anti-convulsive treatment, the maintenance of normal blood glucose and electrolyte levels, and the management of blood count abnormalities (145, 146). Hypoxia (91) and hyperoxia(147), hypocapnia (148), hyperglycemia (149), hypotension and hypoperfusion (124), metabolic acidosis, and seizures (150) have all been documented to aggravate perinatal HI brain injury (146, 91). Therefore, careful management of these parameters is essential in the first week after perinatal asphyxia. In the first 48 h of life enteral feedings should be withheld due to an increased risk of necrotizing enterocolitis, however, sufficient caloric intake should be established as soon as possible (151, 91).

Research data indicates, that the most critical time window for neuroprotective intervention is the first 6 hours after birth, as this is likely the time between the initial HI insult and resuscitation and the secondary brain damage (146, 152). In the current clinical practice, hypothermia is the only neuroprotective treatment, which specifically targets the consequences of HI brain injury and has been proven to be beneficial in large clinical trials (146). Following the TOBY trial, published in 2014, hypothermia has become the standard of care for term neonates with perinatal asphyxia and consequent HIE. Authors found, that total body moderate hypothermia, did not alter combined rates of death and severe disability, but improved neurological outcome in survivors at 18 months (130). However, according to a Cochrane review published in 2013 of 11 randomized control trials with therapeutic hypothermia, moderate hypothermia also reduced mortality in term and late preterm newborns with HIE (153). There is evidence, which indicates, that treatment with moderate hypothermia after perinatal asphyxia led to improved neurocognitive outcome at school-age, however, the opposite has also been reported (130, 154). In the current clinical practice, therapeutic moderate hypothermia is induced within the first 6 h of life and is maintained for 72 hours (146). There is also evidence, that hypothermia initiated before 3 hours of life yields further benefits regarding outcome (155). The neuroprotective effects of hypothermia include the suppression of cerebral

metabolism, the reduction of excitatory neurotransmitter and free radical release, the inhibition of apoptosis, seizure prophylaxis and the amelioration of the neuroinflammatory response (146).

In the past decades, novel neuroprotective strategies have been the primary focus of research in the field of perinatal HI brain injury (156). Many neuroprotective agents are being investigated, which either target the excito-oxidative cascade or the neuroinflammatory response. Blocking the inflammation after perinatal HI injury appears to confer neuroprotective effect, which could yield important therapeutic benefits (157-160). So far, few pharmacological agents proved to be effective in human preclinical trials (161). One of the most promising agents is erythropoietin (EPO), which has pleiotropic effects, such as neuroregenerative, angiogenic, anti-inflammatory, and antiapoptotic effects (162-165, 161). Neurons, astrocytes and microglial cells all express EPO receptors, upregulate its expression upon HI insult and neuroinflammation (165) and EPO has been shown to exert anti-inflammatory and neuroprotective effects (166). A pilot trial of recombinant human EPO (rhEPO) in perinatal asphyxia showed that treatment resulted in improved EEG background activity and decreased serum NO levels at 2 weeks of age and fewer neurologic and developmental abnormalities at 6 months of age (167). However, in NAIS rhEPO treatment did not alter the extent of the brain infarction or neurodevelopmental outcome (168). Phase II and III clinical trials are currently in process (165). The administration of melatonin is also currently being assessed in clinical trials (NCT02621944). Melatonin also has pleiotropic effects, targeting several steps of the neuroinflammatory cascade and has been shown to exert antioxidative, free radical scavenging and anti-inflammatory effects (169, 170, 161). In piglets, administration of melatonin together with hypothermia decreased neuronal loss and preserved function (171). Xenon is another promising neuroprotective agent, showing synergic neuroprotective effects when combined with hypothermia (172). It is an antagonist of the NMDA glutamate receptor (173) exerting its protective effect primarily via inhibiting intrinsic apoptotic pathways. It has a very low blood-gas partition coefficient (174) enabling it to pass the through the BBB quickly. Xenon has been shown to reduce neuronal injury (175). There are ongoing clinical trials investigating the efficacy of hypothermia combined with xenon inhalation (NCT01545271). Doxycycline is an

important inhibitor of neuroinflammatory response, which reduces of IL-1β and TNF-α expression and is approved for use in neonates, has few side effects, thus making it a promising candidate (176). Histone deacetylases exert pleiotropic anti-inflammatory and neuroprotective effects, via inhibiting the components excito-oxidative cascade, inhibition neuroinflammation and proapoptotic factors (177-179, 161). Further promising anti-inflammatory and neuroprotective agents include selective cyclooxygenase-2 inhibitors, resveratrol, N-acetylcysteine, TNF-α soluble receptor, IL-1ra and magnesium, which acts as an (endogenous cationic NMDA channel blocker) (180, 161).

One intriguing recent discovery is, that mesenchymal stem cell (MSC) and neural progenitor cell therapy can protect the developing brain from HI injury. The hypothesis is, that MSCs are either able to “migrate to the site of injury, differentiate into specific cell lineages and exert anti-inflammatory effects” or stimulate the differentiation of endogenous precursors, inducing neuroregeneration and plasticity (77, 2). The strenuous efforts to find novel, individualized anti-inflammatory and neuroprotective strategies clearly outline the paramount importance of this aspect of managing neonates with perinatal HI brain injury. Overall, the past decade has brought much insight into the underlying pathomechanism of perinatal HI injury and potential ways to counteract the detrimental consequences, however, continuous efforts in this field are essential in the future as well.

2.2.7 OUTCOME

In developed countries, perinatal asphyxia continues to be a great burden on society as a leading cause of neonatal mortality and neurodevelopmental disability. Mortality and morbidity rates vary among the literature, but according to a recent review on outcome by Antonucci et al., in term neonates, the mortality rate is around 20% and about 25% of survivors will develop neurological disabilities after perinatal asphyxia (146, 181, 182). Another review assessed the prevalence of individual neurodevelopmental disabilities after perinatal asphyxia and found, that of all children with adverse outcomes, 45% sustained "cognition and developmental delay or learning difficulties, 29% cerebral palsy, 26% blindness or vision defects, 17% gross motor and coordination problems, 12% epilepsy, 9% hearing loss or deafness, and 1% behavioral problems" (146, 183). A follow-up study by Van Handel et al. however, revealed, that when assessing at school-age, behavioral, social and attention problems were much more common in children, who sustained perinatal asphyxia (184). If neurological outcome was assessed in correlation with the initial clinical stage of HIE, mild insult lead to no serious neurodevelopmental alterations, whereas cognitive impairments and/or sensory-motor impairments or death occurred in 32% of children who initially had moderate HIE and 100% of children with severe initial HIE (185). However, further studies revealed, that subtler cognitive deficits and behavioral problems were also present following mild HIE (186). It appears, that the outcome is the most variable and the most challenging to predict in neonates with moderate HIE (146, 187, 188).

A recent meta-analysis aimed to assess the prognostic value of currently available clinical diagnostic methods in patients with perinatal asphyxia and HIE. Data was collected from 29 studies and they found that overall, clinical and neurological examination and cerebral ultrasound had poor predictive value. In the first week of life, aEEG (sensitivity 0.93, specificity 0.90), conventional EEG (sensitivity 0.92, specificity 0.83), and VEP (sensitivity 0.90, specificity 0.92) had the best prognostic value regarding neurological outcome. From the imaging techniques, if performed in the first week of life, diffusion weighted MRI had the highest specificity (0.89), whereas T1/T2-weighted MRI (performed within the first 2 weeks of life) had the highest sensitivity (0.98). Early MRS had a sensitivity of 0.75 and poor specificity (0.58) (189). The data of this meta-analysis is summarized on Figure 6.

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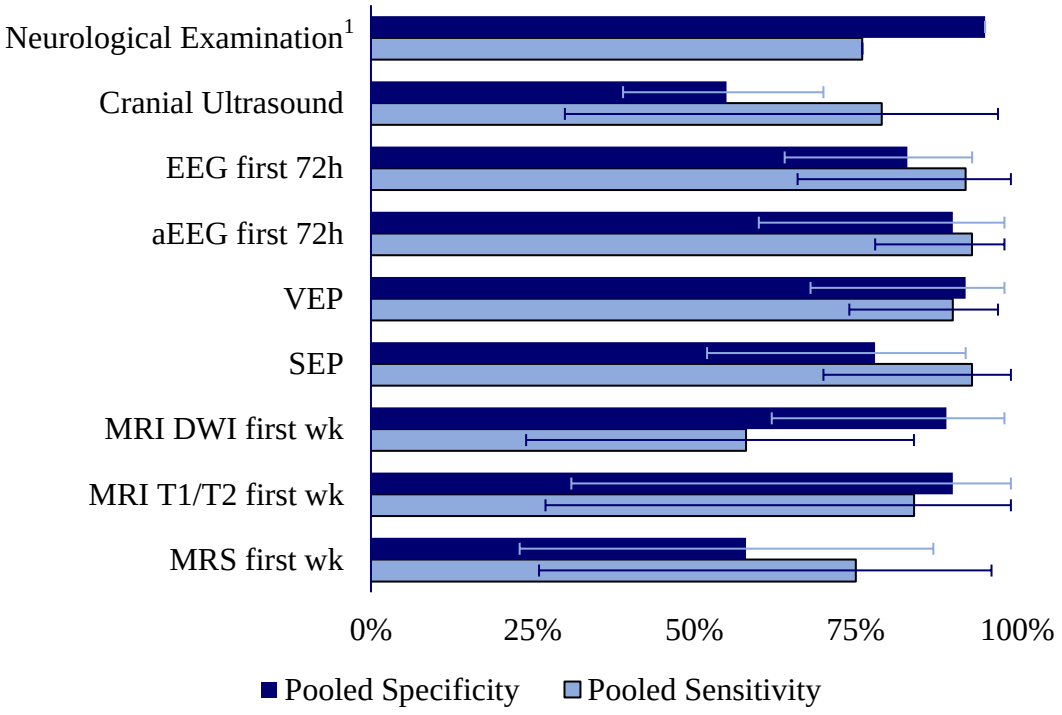

Figure 6. Prognostic value of the diagnostic methods available in the clinical practice. *The original data which this figure is based on was published by van Laerhoven et al. (189) Pooled specificity and sensitivity values with confidence intervals are shown for each diagnostic test. [1]The sensitivity and specificity of neurological examination is based on data from a single study (190). EEG = Electroencephalogram, aEEG = Amplitude-integrated Electroencephalogram, VEP = Visual evoked potential, SEP = Somatosensory evoked potential, MRI DWI = Magnetic resonance imaging, Diffusion-weighted imaging, MRS = Magnetic resonance spectroscopy.*

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2.3 NEONATAL ARTERIAL ISCHEMIC STROKE (NAIS)

2.3.1 DEFINITION AND INCIDENCE

The perinatal period carries the highest risk for stroke in the entire childhood, with almost similar incidence to that in the elderly population. “Perinatal stroke” is a broad term defining a group of heterogeneous cerebrovascular events which occur between 28 weeks of gestation and 7 days of age. However, many studies discuss the perinatal and the neonatal period together, extending inclusion to the first 28 days of life and the variation in terminology can be somewhat confusing. Although this definition also includes in utero stroke, most perinatal stroke events are thought to occur within a few hours to a few days post-delivery (191).

Neonatal stroke can be categorized based on the distinct characteristics of the lesion. The most common subtypes in term neonates are neonatal arterial ischemic stroke (NAIS), primary Heamorrhagic stroke and cerebral sinus venous thrombosis (CSVT). Heamorrhagic events associated with prematurity, especially intraventricular hemorrhages account for a significant proportion of neonatal cerebrovascular events, however, we only included term neonates in our studies, therefore these events will not be discussed in detail. NAIS by definition is an arterial ischemic stroke, with clinical symptoms occurring in the neonatal period (within the first 28 days of life) which are supported by radiological evidence of a focal arterial infarction. (192-194). Many cases of perinatal stroke are only diagnosed later in infancy, with no evidence of an acute neurological event during the neonatal period. “Presumed perinatal stroke” refers to cases, where the diagnosis is made after 28 days of age, when NAIS most likely remained undetected in the acute phase (191).

The exact incidence of neonatal stroke is difficult to determine and estimates are based on studies of neonatal seizures and population-based studies of childhood stroke (195). The incidence of neonatal stroke is estimated to be around 1/4000 live births, with NAIS occurring in 1/8000 live births.

NAIS generally occurs in term neonates and presents a major risk for life-long motor, cognitive and behavioral disabilities ranging from fine motor impairments to unilateral cerebral palsy, which develops in around 20-30% of affected neonates. Thus NAIS is a leading cause of cerebral palsy (196).

2.3.2 RISK FACTORS

The predisposing factors of NAIS are still under strong investigation and there is little consensus among field experts. There are several challenges in identifying the truly relevant risk factors and their possible impact on the course of AIS in neonates. Firstly, the number of studies is relatively low and those which are available are mostly case reports or small case series, only very few included enough cases to be representative. Secondly, the enrolling criteria and the recorded data are not homogenous, many studies include AIS and borderzone infarction as well and some do not distinguish between neonatal stroke cases and stroke occurring in older children (192). Another important challenge is the overlap of the risk factors and presentation NAIS with perinatal asphyxia. Furthermore, in the case of certain risk factors, similarly to HIE, it is very difficult to determine their place in the causal pathway. Authors Lee et al. propose the following possibilities for the role of factors such as fetal heart rate abnormalities in the development of NAIS (197):

1) Plays a primary causal role
2) Is on the causal pathway between preceding factor and NAIS
3) Adverse effect of causal factor with no impact on NAIS
4) Direct consequence of NAIS
5) Combination of the above

Further such factors include prolonged second stage of labor, vacuum assistance, and emergency cesarean section. These factors show a clear association with NAIS cases, however the impact of these factors is yet to established, and this must be considered when interpreting findings. Overall, there is little consistency among the different studies (193). Therefore, instead of true risk factors, it is more correct to discuss “associations”, which have been documented in numerous studies (Table 3).

Table 3. Factors associated with elevated risk of neonatal arterial ischemic stroke (NAIS). *The data were collected from the latest report of the largest database, the Canadian Pediatric Ischemic Stroke Registry (CPISR) and an American case-control study published in the Journal of the American Medical Association (JAMA).* [1] *significant association in the CPISR (percentage of neonates affected),* [2] *significantly difference compared to controls in the JAMA case-control study (198, 197).*

Maternal factors	Fetal factors
Antepartum	
Primiparity[1,2] (38.33%)	Oligohydramnios[2]
Diabetes/ Gestational Diabetes[1] (3.89%)	Decreased fetal movement[2]
Hypertension[1] (7.22%)	Congenital heart disease[1] (12.28%)
Preeclampsia[1,2]	Prothrombotic disorders[1] (12.72%)
Infertility[1,2] (1.11%)	
Bleeding[1] (3.89%)	
Streptococcus positivity[1] (6.11%)	
Smoking[1] (13.89%)	
Alcohol[1] (7.22%)	
Recreational Drug Use[1] (3.33%)	
Acute Viral Illness[1] (3.33%)	
Antibiotic Use[1] (3.33%)	
Chorioamnionitis[1,2]	
Intra-/ postpartum	
Pyrexia[1] (16.67%)	Fetal heart rate abnormality[2]
Premature Rupture of Membrane[1] (10.56%)	Cord abnormality[2]
	Bacterial sepsis[1] (8.33%)
Placental abruption[1] (1.67%)	Meningitis[1] (3.95%)
Prolonged second stage of labor[2]	Shock[1] (2.63%)
Vacuum assistance[2]	
Emergency section[2]	

According to the latest data from the Canadian Pediatric Ischemic Stroke Registry (CPISR), which is the largest database available to date, an identifiable risk factor was only present in 56% of neonates with stroke, while the rest of the NAIS cases were considered to be of idiopathic origin. Furthermore, 21% of neonates with AIS also presented with asphyxia at birth (198). The identifiable risk factors of NAIS however appear to be specific for the perinatal period and can be distinguished from the risk factors of stroke in older children or adults (192). Most NAIS cases are believed to be of multi-factorial origin, in an American case-control study, the presence of three or more risk factors increased the risk of perinatal stroke 25-fold (197), therefore identifying predisposing factors has been in the limelight of research.

One independent risk factor, which was found relevant in almost all studies was perinatal inflammation (196). Several markers of perinatal inflammation appear consistently as risk factors for NAIS, most importantly maternal fever and neonatal infection. In the CPISR bacterial sepsis, shock or meningitis was found in altogether 15% of NAIS cases, and there is consequent documentation of arterial ischemic stroke due to focal arteritis as a classical complication of bacterial meningitis (198-202). In the CPISR, maternal fever was documented in 17% and premature rupture of membranes in 11% of NAIS cases (198). Chorioamnionitis in the absence of fetal distress was significantly associated with NAIS (197). Maternal fever is a classic indicator of chorioamnionitis, however, most studies did not include histological examination of the placentae, since NAIS is typically diagnosed within days after delivery. Studies, which are available indicate a strong association between NAIS and histological chorioamnionitis (203, 197). The need for large-scale placental tissue banking to understand the contribution of antenatal exposure to inflammation to the pathogenesis of NAIS has been raised by experts (193).

Further associated maternal risk factors include primiparity (38%), gestational diabetes, hypertension, preeclampsia (13%) (198), thrombophilia, including antiphospholipid syndrome, history of infertility, smoking and drug use. 24% neonates were delivered by Caesarean section. Many neonates with AIS needed supportive care following birth, 39% of neonates needed supplemental oxygen, 12% required bag and mask ventilation, 18% had to be intubated and in 7% of all cases a complete cardiopulmonary resuscitation was necessary (198).

Among fetal risk factors, one of the most prominent is congenital heart disease which was present in 12% of NAIS patients according to the CPISR. There has been much interest in the role of genetic prothrombotic disorders in the etiology of NAIS, and several studies show a positive correlation between NAIS and different prothrombotic states (204). A meta-analysis of observational studies on prothrombotic disorders found thrombophilia to be a risk factor for both AIS and sinovenous thrombosis in all age groups during childhood (205). However, studies based on clinical thrombophilia testing found less correlation and raised awareness to the challenges and variety of laboratory methods (206). Other studies debate the relevance of prothrombotic disorders, arguing that most studies which linked genetic thrombophilia to NAIS were based on a heterogeneous cohort of term, late preterm, and early preterm neonates, thus making data less reliable (196, 201). The impact of thrombophilia on the incidence and recurrence of NAIS remains a focus of investigation, however, it appears to be clear, that thrombophilia alone can rarely account for all the cases of perinatal stroke (193).

2.3.3 PLACENTAL PATHOLOGY

Among other risk factors, pathological placental alterations have been implemented to contribute to the development of perinatal stroke. In one case-series authors aimed to systematically characterize placental pathology along the assessment of maternal and fetal risk factors in 5 NAIS and 7 CSVT cases (203).

In their cohort, 10 out of 12 neonatal stroke patients (83%) had pathological placental alterations, and in several cases, there was evidence of multiple types of lesions. Most patients also had several prenatal or neonatal risk factors, supporting the theory, that neonatal stroke might be the end result of a combination of predisposing and triggering factors. 50% of neonates with stroke had lesions consistent with thromboinflammatory processes, such as fetal thrombotic vasculopathy, acute chorioamnionitis, chorionic vessel thrombosis, stem vessel thrombosis or umbilical vein thrombosis, which could serve as the source of cerebrovascular thromboembolism, although the impact of such alterations is difficult to determine without control data. Fetal thrombotic vasculopathy is a disorder, when thrombosis develops in the fetal side of the placental circulation and it has been associated with

systemic neonatal thromboembolism, including cerebral embolism (207). Histologic evidence of a sudden catastrophic event, such as retroplacental hematoma, umbilical vessel occlusion or umbilical cord entanglements was also present in nearly half of the cases. Authors hypothesized that the blood stasis could lead to clotting and umbilical vessel thrombosis, which could be a source of emboli.

Placental lesions indicating decreased placental reserve were present in 3 cases. Placental insufficiency and infection are known to activate the inflammatory cascade and were shown to increase the production of pro-inflammatory cytokines such as IL-6, IL-8, and TNF-α in response to vascular compromise and oxidative stress (208-210). Decidual cytokines have been shown to pass through the placental membranes and appear in the fetal circulation, and in clinical chorioamnionitis, elevated concentrations of cord-blood cytokines have been associated with neurological symptoms, such as seizures (211, 212). In vitro stimuli of hypoxia and growth restriction have been observed to induce TNF-α production leading to fibrin deposition in placental villi, which is able to alter the endothelium to create a thrombogenic surface and thus activate the coagulation cascade (213, 203). The role of the activated inflammatory and coagulatory cascade in the development of ischemic neonatal brain injury is supported by much of recent years' research and remains a central topic of interest. Authors concluded, that the placenta could serve as an important source of embolism and cytokines and pathological alterations could contribute to the development of perinatal stroke (203).

2.3.4 PATHOPHYSIOLOGY

Different neonatal cerebrovascular events may have distinct etiological backgrounds. NAIS by definition is an arterial infarction, which is assumed to be the result of a thromboembolic event leading to a focal ischemic lesion in the area of the affected cerebral artery. The thrombi are presumed to originate from intra- or extracranial vessels, the heart or the placenta (192). The pathophysiology of NAIS is poorly understood and disease specific preventive measures, prognostic factors and therapeutic strategies are not available (193).

Interestingly, NAIS almost always occurs in intra-cranial arteries developing from the carotid arterial tree, i.e. the proximal parts of the anterior cerebral artery

(ACA), the middle cerebral artery (MCA) and the posterior cerebral artery (PCA)—while the basilar artery and the extra-cranial arteries are left unaffected (214-216). Similar to adults, the area of the MCA is the most common localization of the ischemic lesion in neonates as well, with 83% of focal events affecting this area, of which 66% are left sided, resulting in a higher incidence of right sided congenital hemiplegia (217, 194).

According to the classic pathophysiological hypothesis the origin of the thrombi are proximal sources such as the placenta and the umbilical vessels (191, 214). The thrombi are supposed to undergo right to left shunting via the patent foramen ovale and lead to cerebral embolism and ischemic lesions, which are often multifocal in NAIS. The pathological findings in NAIS are easy to differentiate from disorders connected to decreased cerebral perfusion, such as border zone ischemia, which is usually bilateral and localized to the area between two major vessel territories (192). It appears, that placental abnormalities might play an important role in the development of thromboembolism in the neonate. The recurrence rate of NAIS is almost zero, supporting theories that emphasize the role of factors only present perinatally (193). There are data from small clinical studies, that indicate a direct correlation between pathological placental alterations, especially placental inflammation or chorioamnionitis and NAIS (203, 197). Maternal and fetal/neonatal inflammatory biomarkers are currently under strong investigation with the hope, that identifying key inflammatory signatures may help in gaining a better understanding of the disease pathology and might open the way for better prognostic and diagnostic strategies (193).

Although there is evidence supporting the role of placental inflammation in the development of NAIS (193), they can only be identified in some of the cases and this hypothesis does not give a plausible explanation to why NAIS is almost exclusively affecting the intracranial arterial territories developing from the carotid arterial tree, while the incidence of basilary arterial or extracerebral infarcts is negligible (191). In recent years, angiographic alterations were described, raising the notion that local arterial wall defects and in situ thrombus generation might also play an important role in the development of NAIS (218, 206). Guiraut et al. hypothesized that maternofetal inflammation could induce focal arteritis specifically in the intracranial arteries

developing from the carotid arterial tree, which are susceptible to NAIS (196). Using a preclinical rat model of chorioamnionitis, they were able to demonstrate, that classic prothrombotic stress on the MCA, when applied alone was not enough to induce NAIS, however, when combined with in-utero exposure to inflammation inducing agent lipopolysaccharide (LPS), was able to lead to the classical symptoms of NAIS and motor impairment. They also examined the walls of arteries susceptible to NAIS and found that the constitutive expression of certain pro-inflammatory cytokines, i.e. TNF-α and IL-1β was higher in the susceptible intra-cerebral arteries than in extra-cerebral arteries. Furthermore, pups born from LPS-exposed dams developed a specific cerebral arteritis with increased presence of inflammatory cells (macrophages) and elevated levels of pro-inflammatory cytokines IL-1β, TNF-α, MCP-1 and increased IL-1/IL-1 receptor antagonist (ra) ratio in NAIS susceptible arteries, but not elsewhere (214).

There is further evidence supporting the role of prenatal inflammation in sensitizing the brain for postnatal HI injury (219-221). The initial hypoxic insult leads to primary energy failure, oxidative stress and excitotoxicity resulting in widespread necrosis and necroptosis within the first 6 hours of the insult (222-224). Neural tissue damage could be aggravated by previous sensitization due to inflammation, which could lead to increased oxidative stress and an increased production of pro-inflammatory cytokines (225, 220, 226-229).

Based on these findings a novel pathophysiological hypothesis is being raised, which considers the complex and bidirectional relationship of coagulatory and inflammatory pathways (230). According to it, the development of NAIS can be described as the result of “multiple hits”, originating from both perinatal inflammation and hypoxia–ischemia (HI) (196). Guiraut et al. have identified three major risk factors, the combination of which could lead to NAIS: (i) materno-fetal inflammation leading to focal arteritis in arteries susceptible to NAIS, (ii) a window of susceptibility in the development of the neonatal brain and (iii) the physiological pro-coagulatory state of the perinatal period aggravated by the inflammatory response (214).

The role of the inflammatory pathway in the development of an ischemic brain injury is supported by much of recent years’ research data. Not only does it appear to be a critical step in the pathomechanism of neonatal stroke, but the subsequent

neuroinflammatory response to the ischemic brain injury is also a common feature of perinatal asphyxia and NAIS. The most important aspects of the neuroinflammation are the production of inflammatory cytokines, the migration of leukocytes into the brain tissue leading to necrosis and apoptosis. T lymphocytes appear to play a central role in the development of an ischemic brain infarct, CD4+ (Cluster of Differentiation) and CD8+ T cells migrate to the CNS already a few hours after a HI injury. The prevalence of T lymphocytes reaches its maximum around 3-4 days after the HI injury and the inhibition of T cell trafficking to the CNS ameliorates the detrimental effects of neuroinflammation after AIS (231, 232). T cell deficiency has also been shown to reduce infarct size and improve neurological outcome in murine ischemic stroke models (233-235).

Human experimental data on the role of inflammatory markers in shaping the course of NAIS is scarce, one small case-control study aimed to describe the levels of different cytokines in the plasma of pediatric AIS patients. Elevated levels of TNF-α, IL-2, IL-6, and IL-8 were observed 6 months following AIS compared to healthy controls. No alterations were present in soluble endothelial protein C receptor, IL-11, and FVIII median levels (236). The limitation of this study is that they included both neonatal and pediatric stroke cases (from birth until 18 years of age), which are now recognized as distinct clinical syndromes. However, these results indicate that an ongoing inflammation could be observed up to 6 months following AIS, although, no information is available regarding the acute phase of NAIS. Many studies have been focusing on the immunological consequences of AIS in adults and several cytokines have been suggested to play an important role in the development of the ischemic lesion. Pro-inflammatory cytokines IL-1β, IL-8, MCP-1, TNF-α and IFN-γ appear to exacerbate cerebral injury, whereas anti-inflammatory cytokines such as TGF-β and IL-10 appear to be neuroprotective (237-239). Although the immune system of neonates differs from that of adults on many accounts, the inflammatory network appears to play a similarly critical role in the course of an ischemic brain injury. Identifying the key players of the inflammatory and apoptotic pathways is a cornerstone of current research focusing on NAIS and neuroinflammation, since the time period after a hypoxic injury could be important for targeted intervention (233, 240, 196, 241).

2.3.5 CLINICAL PRESENTATION AND DIAGNOSIS

The greatest challenge in the diagnosis of perinatal stroke is that unlike in older children and adults, the clinical presentation in neonates is often subtle and non-specific (192). Neonates rarely present with focal signs such as unilateral motor deficit. The most common clinical manifestation is focal or generalized seizures in the first 12-72 hours of life, which are estimated to occur in 70-90% of neonates. According to the Canadian Stroke Registry, neonates with stroke presented with seizures in 88% of the cases, whereas focal neurological deficits, mainly hemiparesis was present in only 12% of the cases (198). Studies focusing specifically on neonatal seizures have raised attention to the incidence of perinatal stroke, which was found in 12-14% of neonates with seizures, making stroke the second most common cause of seizures in term neonates (242, 243). Many neonates also develop generalized encephalopathy, with irritability, hypertonia or lethargy and hypotonia and poor feeding present in 36-63% of cases (191). The neurological examination of NAIS patients usually results in non-specific findings such as general hypotonia and lethargy.

The incidence of NAIS is still hard to determine and many cases remain undiagnosed, since affected neonates may present with very subtle or no symptoms during the neonatal period. Thus NAIS is diagnosed retrospectively in infancy, when the most common signs are early hand preference and delayed motor milestones, however hemiparesis, focal seizures or infantile spasms may also occur. At this time, neuroimaging may reveal signs of chronic focal injury (191, 192).

Another challenge in the timely diagnosis of NAIS is that the risk factors and clinical signs of global hypoxic-ischemic encephalopathy due to perinatal asphyxia show a significant overlap with NAIS and the two often co-occur (206, 193, 244). Differentiating between the two syndromes is a challenging, some studies list perinatal asphyxia as an independent risk factor for NAIS (192), and many neonates develop general encephalopathy after NAIS, while the neuroinflammation following the initial hypoxic insult appears to be a common feature of the two.

Although trends are improving, recent results from the CPISR still indicate delayed diagnosis due to the lack of specific clinical features and the high rate of false-negative results with cranial ultrasound and CT scans as a major challenge in NAIS. Authors emphasize the importance of high awareness to a possible AIS in neonates

and the need for timely MRI scanning (198). The best modality for detecting and characterizing NAIS and differentiating it from diffuse hypoxic-ischemic encephalopathy is MRI with vessel imaging (Figure 7). It is the most sensitive at 2-4 days after the hypoxic injury, however, restricted diffusion on diffusion-weighted imaging can indicate a stroke earlier (191).

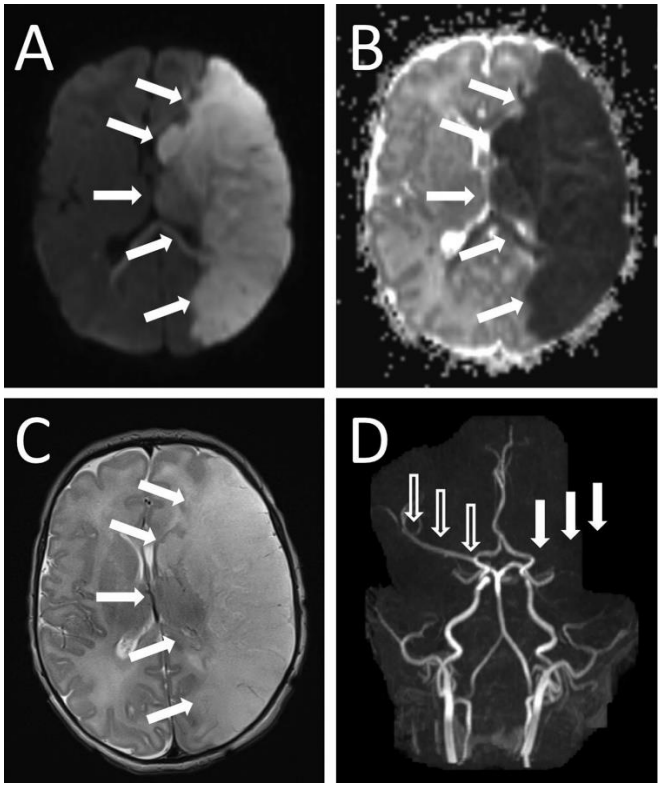

Figure 7. MRI and MRA images of the brain of a term neonate with NAIS. *Images show a large infarct in the area of the MCA. This image was published by Bernson-Leung et al. (191) with the following legend: Term neonate presented with right eye twitching at delivery. Axial images showed restricted diffusion indicating acute ischemia (bright on trace image [A] and dark on ADC map [B]) occupying the entire left MCA territory (arrows). C. Sulcal effacement and obliteration of the gray-matter/white-matter junction are seen on the axial T2 sequence. D. Magnetic resonance angiography showed absent flow signal in the expected location of the MCA due to occlusion (solid arrows indicate expected course as compared to white outlined arrows that indicate course of right MCA). He developed infantile spasms at 5 months. At 13 months, he had a spastic hemiparesis, could sit but not crawl, and had no specific words. MCA = middle cerebral artery.*

2.3.6 THERAPY

The diagnosis of NAIS is often delayed, due to the prenatal onset or absence of specific signs, therefore the primary therapeutic aims are prevention and post-insult anti-inflammatory treatments (196). Currently no disease-specific therapeutic methods are available in the acute care of NAIS patients. Therapy mainly consists of intensive care, circulatory support and seizure prophylaxis. Recurrence rates are very low (1-2%) and occur mainly in cases with identified cardiac abnormalities or coagulation disorders, therefore chronic anticoagulatory or anti-platelet therapy is not recommended (191). The long-term care of these patients is primarily centered around early rehabilitation, with the hope, that the neuroplasticity of the developing brain can be harnessed to minimize neurological deficits. More and more studies are available on the recovery of function after NAIS, providing valuable insight in the mechanisms of recovery in the immature brain (193).

A central question regarding anti-inflammatory therapeutic methods following NAIS is the potential value of therapeutic hypothermia, which is currently the standard of care for term neonates with perinatal asphyxia resulting in HIE. There is substantial evidence supporting the role of hypothermia in reducing mortality and improving neurological outcome in neonates with hypoxic ischemic encephalopathy (130). Hypothermia has been shown to reduce infarct size in animal models of focal cerebral ischemia (245), decrease cytokine levels in HIE (246) and there is evidence that hypothermia might lower the risk of seizures after perinatal stroke and thus improve outcome (247). There is currently an ongoing discussion within the scientific community about extending the indication of therapeutic hypothermia to include new categories, such as preterm neonates with HIE and perinatal stroke, however there are still many concerns which need to be clarified before new therapeutic methods can be introduced into the clinical practice (233, 245).

2.3.7 PROGNOSIS AND OUTCOME

Disease specific prognostic markers are currently not available for neonatal stroke. Data analysis from the largest database, the CPISR revealed, that certain aspects of the clinical presentation and the course of NAIS can be associated with poor outcome. Predictors of poor outcome were decreased level of consciousness,

nonspecific presentation and basal ganglia stroke location. Posthoc analysis of correlation between clinical presentation and outcome showed that neonates presenting with cardiorespiratory symptoms such as apnea or cyanosis had the highest chance for poor outcome (198). Abnormal EEG during the first post-stroke week and internal capsule involvement were associated with the development of hemiplegia (248). Infants diagnosed following the neonatal period also had a higher chance for adverse neurological outcome (192, 249).

Neonatal stroke is still a major cause of disability in the developed countries. Data from the CPISR indicates, that only 40% of neonates who suffered stroke show a normal long-term normal neurological outcome. The mortality rates in the acute post-stroke phase have reduced to 9%, however, long-term disability is still a great issue. Early outcome data showed, that 70% of neonates showed no deficits at the time of discharge, 20% showed mild, 6% moderate and only 2% severe neurological deficit. On the contrary, long-term follow up revealed, that neurological deficits increased from 30% to 60% among neonates, with 39% of neonates showing a worsening neurological function over time, while only 6% showed improvement. Late seizures occurred in 17% of neonates. This clearly shows one of the important challenges in the long-term care of neonatal stroke patients, which is the emergence of novel neurological deficits with time. These deficits could indicate the role of chronic neuroinflammation in long term functional recovery, however could also be a result of the initial insult, with the symptoms manifesting only when the child's neurodevelopment reaches a certain point (for example in the case of speech deficits) (250). Long term outcome data showed mild deficit in 33% of cases, moderate in 19% and severe deficit in 8% of cases. Interestingly, basal ganglia stroke in neonates was associated with a four-fold increase in the chance of poor outcome, which could be due to the disruption of the developing neural networks necessary for cortical migration in the periventricular zone of the immature brain of neonates who suffer subcortical infarcts (251). Authors argue, that the high rate of adverse neurological outcome supports the vulnerability hypothesis and not the enhanced plasticity hypothesis regarding the recovery after neonatal stroke (252, 198, 253, 254).

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2.4 NEUROINFLAMMATION

2.4.1 HYPOXIA-INDUCED NEUROINFLAMMATION

Inflammation of the CNS or neuroinflammation is now recognized to be a common consequence of hypoxic brain injuries, including perinatal asphyxia and stroke. The brain injury following perinatal hypoxia-ischemia evolves over time as a result of several mechanisms, however neuroinflammation is considered to be a major pathogenic factor (180). The inflammatory response is not limited to the CNS, but can also be detected systemically (255, 256). The level of immune activation and the complex neuro-immune cross talk appear to determine the severity of the brain damage, influencing long-term outcome.

The neuroinflammatory response can be divided to three phases, an early primary, a delayed secondary and a chronic tertiary phase. The initial insult leads to insufficient glucose delivery to the highly sensitive developing brain tissue, which triggers a metabolic chain reaction resulting in the exhaustion of the energy stores of the neuronal tissue (primary energy failure). This, along with the direct consequences of cellular hypoxia leads to extensive cellular damage (50, 257, 161). Inflammatory signals are released within minutes, leading to microglial activation followed by systemic immune activation. Microglia and astrocytes release pro-inflammatory cytokines and chemokines leading to the disruption of the BBB, allowing peripheral leukocytes to migrate to the CNS. In comparison with adults, in whom leukocyte infiltration and cytokine production follows microglial activation within hours, neonatal immune responses are triggered almost immediately after cerebral ischemia and continue for weeks (258, 259, 255).

The second phase is initiated after approximately six hours and is mainly characterized by the excessive production of pro-inflammatory cytokines, chemokines, NOS, ROS, RNS, excitatory amino acid agonists, and death receptor agonists by both the activated (intrinsic) microglia and the infiltrating monocytes and T cells (260-262). This leads to excitotoxicity, apoptosis and further necrosis resulting in “delayed neuronal death” (112, 69), which is responsible for a large percentage of the total neural cell loss regardless of the severity of the initial hypoxic-ischemic insult. Further contributing factors to the brain tissue loss include axon and myelin injury, the

loss of oligodendrocyte progenitors and mature oligodendrocytes (263).

The tertiary phase of brain damage is defined by authors Fleiss and Gressens as a collective result of those processes, which “worsen outcome, predispose a patient to further injury, or prevent repair or regeneration after an initial insult to the brain” (Figure 8) (118). They propose, that the tertiary phase might persist for months or years after the initial injury, contributing to the long-term consequences of neuroinflammation. Their aim is to raise awareness to the notion, that there might be active processes long after the initial brain injury, and that this tertiary, latent phase of neuroinflammation would be a crucial time for targeted intervention. Therapeutic aims include the regulation of the sustained microglial activation and inflammatory processes and the modification of adverse epigenetic alterations. Although the data from animal models and studies on adults are intriguing, little is known about the impact of these tertiary processes on neurodevelopmental outcome in neonates. Learning more about the events of this chronic phase of neuroinflammation in human perinatal HI brain injury could open new possibilities for individualized therapy and targeted intervention to improve long-term neurological outcome (118).

According to the hypothesis of Fleiss and Gressens the key features of this phase are:

1) Persistent inflammation
2) Aberrant gliosis
3) Epigenetic changes
4) Consequent blockade of oligodendrocyte maturation
5) Sensitization to further injury (264, 118, 265)

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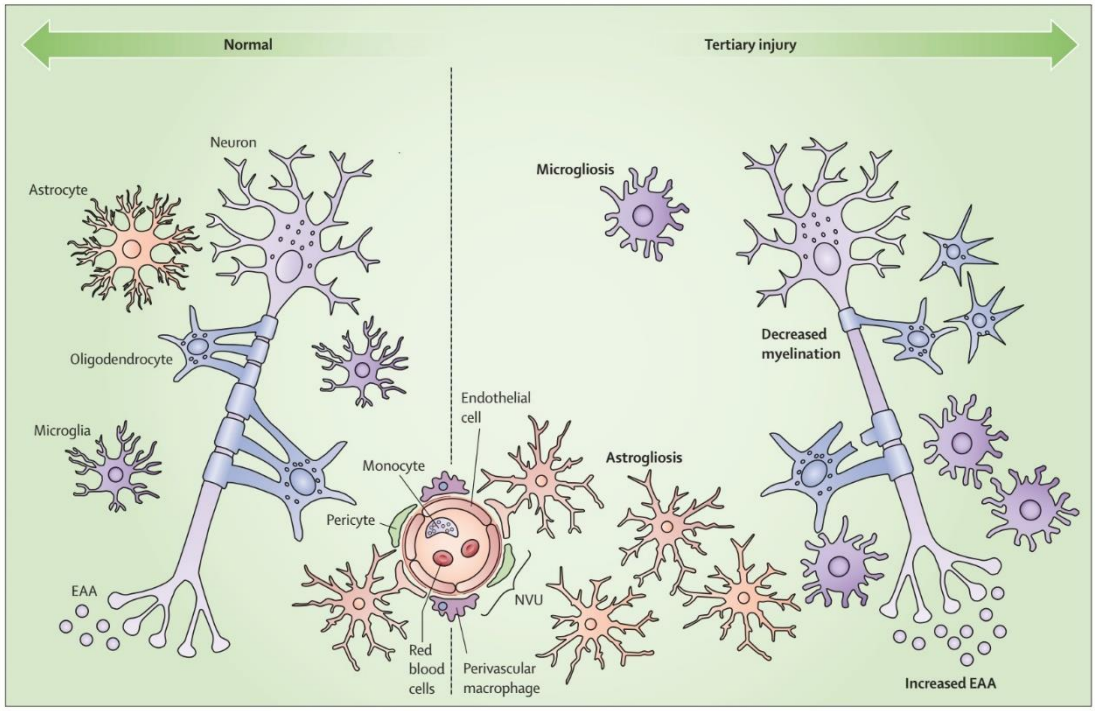

Figure 8. Schematic image of the key features of the tertiary phase of neuroinflammation. *This image was published by Fleiss and Gressens (118) with the following legend: Changes in oligodendrocytes, maturational blockade, and the production of glial scar products leading to decreased myelination, changes to glia, astrogliosis and microgliosis, and possible changes in EAA bioavailability are shown. Also shown is the NVU, a possible target for novel therapeutics because of its ability to mediate transmigration of inflammatory mediators. EAA= excitatory aminoacid. NVU= neurovascular unit.*

1) Persistent inflammation

A growing body of evidence indicates the presence of persisting neuroinflammation long after the initial brain injury. In non-human primates microglial activity and cytokine production was increased 12 months after traumatic brain injury (266). Human in vivo data from PET (positron emission tomography) scans indicate persisting microglial activity 10 years after traumatic brain injury, which correlates with worse neurological outcome (267). T lymphocytes and other inflammatory cells and signals can be detected for months following the initial HI brain insult (268) Microglial cells also play a key role in the maturation and

development of the CNS and authors Fleiss and Gressens propose, that sustained microglial hyperactivity could also have an impact on synaptic pruning, neurogenesis, pathway excitability and memory, which could predispose the brain to cognitive impairments (118).

2) Reactive gliosis

Reactive astrogliosis is a key protective mechanism during the acute phase of HI brain injury, which limits the leaking of the BBB by creating a barrier around the site of injury (118). Astrogliosis is a key feature of the long-term consequences of HIE. Activated astrocytes and components of glial scars have been shown to inhibit axonal regrowth and remyelination, inhibiting regeneration and repair processes even months after the initial phase (118, 269-271) Astrocytes also play a key role in regulating the level of neurotransmitters, and decreased glutamate transporter expression has been shown to persist until at least 21 days after perinatal HI brain injury in rats (272), which could play a role in sustaining long-term excito-oxidative processes.

3) Epigenetic changes and consequent inhibition of oligodendrocyte maturation

Epigenetic modifications are by definition “the enzymatic changes of transcription via the modification of permissive tags on histones or DNA and microRNA-mediated alterations of translation” (118). Epigenetic alterations therefore play a pivotal role in the normal development of the brain and they have been shown to be a key factor in transmitting the long-term consequences of brain injury, “such as cognitive, motor and behavioral impairments” (118, 273). Evidence indicates, that these epigenetic changes are associated with for example cognitive decline, after traumatic brain injury (274, 275). Authors emphasis of the role of epigenetic changes in delayed oligodendrocyte maturation and consequent white matter injuries (118). Recently Favrais and Fleiss et al. have also been able to demonstrate, that chronic perinatal inflammation induced by exposure to IL-β in the first days of life lead to oligodendrocyte maturation failure persisting until adulthood and resulting in behavioral changes (264).

4) Sensitization to further injury

According to Fleiss and Gressens the events of the tertiary phase of neuroinflammation could induce a so-called persistent glial priming, leading to malfunctional pro-inflammatory signaling and alterations in the production of excitatory neurotransmitters, which could sensitize the brain to following injuries (118, 276, 277). In support of this hypothesis, perinatal exposure to LPS and other viral and bacterial mimetics have been shown to reprogram neuroimmune responses and increase the vulnerability of the brain for HI injury and inflammation in the adulthood. This was associated with accelerated cognitive decline and aging (278-280, 221).

Overall, hypoxic-ischemic injury effects the developing brain very differently than the adult brain mainly because of the immaturity of the CNS and the immune system. The main characteristics of the neuroinflammatory response following perinatal HI injury are summarized on Figure 9. The age-specific inflammatory, oxidative and excitotoxic mechanisms make the embryonic and early postnatal brain especially susceptible to hypoxic-ischemic injury and increase the chance of long-term neurological consequences and mental health disorders (50, 257, 161).

The detrimental consequences of neuroinflammation have been extensively studied, however, the aspects of the inflammatory response that are beneficial for the CNS regeneration have only recently been brought to light (281, 282). While excessive neuroinflammation contributes to the loss of oligodendrocytes and demyelination, it has become evident that a certain level of neuroinflammation is necessary for CNS recovery. Beneficial effects of neuroinflammation include neuroprotection, the mobilization of neural precursors for repair and axonal regeneration (283). The challenge is to understand and harness the beneficial aspects of neuroinflammation to promote CNS regeneration, while minimizing its harmful effects (284).

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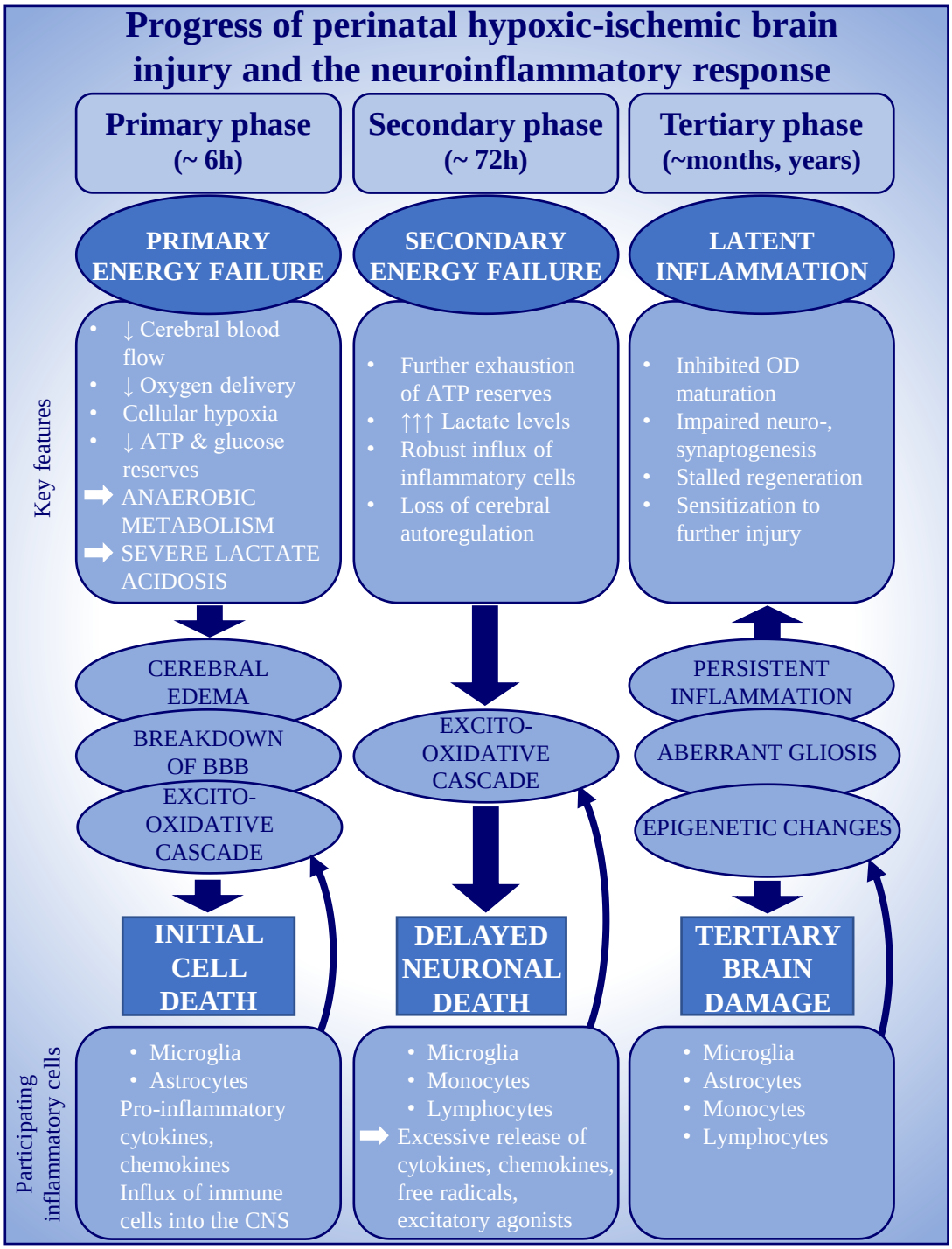

Figure 9. Summary of the progress and development of perinatal hypoxic-ischemic brain injury and the following neuroinflammatory response. *This figure was created from the information in the chapter above, based on the following references: (118, 161) (261-290) BBB = Blood-brain barrier, CNS = Central nervous system, OD = oligodendrocyte.*

2.4.2 CELLS PARTICIPATING IN THE NEUROINFLAMMATION

2.4.2.1 MICROGLIA

The first responding cells to the CNS hypoxia are the microglial cells, the residual immune cells of the brain which are derived from embryonic hematopoietic precursors that enter the CNS before birth and persist until adulthood (285, 286). In resting state, the microglial cells are responsible for immune-surveillance through their ramified processes, which continuously expand and retract to monitor the CNS microenvironment. Upon a hypoxic cerebral insult, they sense the inflammatory signals released from the injured neurons and glial cells and become activated within minutes (287, 288). Following activation, they gain macrophage-like functions such as phagocytosis, cytokine production, antigen presentation and matrix metalloproteinase (MMP) release (257). As a combined result of hypoxia and the inflammatory mediators, the tight junctions of the of the BBB start to break down (289) and large amounts of peripheral leukocytes begin to infiltrate the CNS, aggravating the inflammatory response, which leads to further neuronal injury and apoptosis (284). Depending on the local inflammatory milieu, microglial cells are able to switch between classical activation (M1), characterized by cytokine-, chemokine- and reactive intermediate production and alternative activation (M2), which leads to the clearance of reactive species and anti-inflammatory signaling (290, 291). M1 type of activation is associated with increased neuronal cell loss compared to M2 type activation and thus the phenotype of microglial activation likely plays an important role in setting the course of the neuroinflammatory response (292, 257).

The aggregation of microglial cells in the dental gyrus and the hippocampus appears to be a characteristic result of perinatal asphyxia. In one study, where hippocampal samples from 178 children were assessed retrospectively authors found that neonates who died from HIE had a dense infiltrate of microglial cells in the polymorphous layer of the dentate gyrus, which was missing in infants who died of other acute causes such as sepsis or were older than 9 months (293). Data from animal models of hypoxia-ischemia also support the pivotal role of microglial activation in the neuroinflammatory process. Microglial cells have been shown to accumulate around the hippocampus, the periventricular and the subcortical white matter after

activation due to HI injury in preterm sheep (294). Amoeboid microglial cells on the other hand showed an almost immediate response to HI injury in a murine model, showing aggregation around the brain infarct. These microglial activation lead to the release of pro-inflammatory cytokines, free radical generation and excess glutamate release, which resulted in oligodendrocyte loss, demyelinisation and BBB breakdown (260, 295, 296, 257). Overall, microglial activation appears to be a key factor in the course of the neuroinflammatory response and is in the limelight of research.

2.4.2.2 ASTROCYTES

Astrocytes, along microglial cells are also activated within minutes of a hypoxic cerebral injury. Animal models of HI have revealed, that the astrocytes of a neonatal brain are rather resistant to hypoxia and readily proliferate around the necrotic area (257). Upon activation, astrocytes undergo characteristic changes in their morphological and functional, which results in reactive astrogliosis leading to the formation of glial scars in the affected cerebral region, which appears to be an important feature of perinatal ischemic brain injury (297-299, 161). During the development of the human CNS, astrocytes only become reactive to HI injury from the 20-23rd gestation week onwards (300).

Astrocytes appear to have a dual role in the development of an ischemic brain injury. On the one hand, activated astrocytes are an important source of pro-inflammatory cytokines such as IL-6, TNF-α, IL-1α and β and IFN-γ and neurotoxic substances such as NO, which can aggravate the neuronal damage by inducing apoptosis and inhibiting neurogenesis (301, 302). Reactive astrocytes also play a central role in regulating the migration of immune cells from the periphery to the site of injury via chemokine signals (303, 304). They also induce chemokine and cytokine production in neuroblasts and angioblasts (305). However, there is also much evidence of the neuroprotective role of activated astrocytes, which they exert by secreting anti-inflammatory agents, glutathione and superoxide dismutase (306-308), facilitating extra-synaptic glutamate uptake and thus protecting from excitotoxicity (309-311) and maintaining ion gradients (257, 312, 313). This is further supported by studies which show that in the absence of functional astrocytes, the neuronal injury is aggravated (314, 315). One mechanism of protection might be the conversion of astrocytes into

neural progenitor cells promoting neural cell proliferation, survival, migration and differentiation, which has been shown in adults (316, 161). How the pro- and anti-inflammatory effects of astrocytes are regulated and how this affects the course of a perinatal hypoxic brain injury is however yet to be understood.

2.4.2.3 NEUTROPHILS

The contribution of neutrophils to the neuroinflammation after HI in adults is well established (317, 318). In adults, neutrophils migrate to the site of injury within 6 hours and exacerbate the inflammation by producing ROS and releasing inflammatory agents such as MMP-9 (257). However, neonates appear to have diminished ability to mount neutrophil response to hypoxia-ischemia (257, 161). The migration of neonatal neutrophils through the BBB appears to be impaired (319-321), however, they do appear in the cerebral vessels in the first few hours after HI (260). Furthermore, neutropenia induced before HI was beneficial, while removing circulating neutrophils after the hypoxic event or inhibiting neutrophil function did not bring about any benefits (321, 322). Overall it appears, that neutrophils have a lesser role in the neuroinflammatory process in neonates than in adults (257, 161).

2.4.2.4 LYMPHOCYTES

The role of the adaptive immune system in the development of perinatal hypoxic brain injury is much less studied than that of the innate immune system. The detrimental effect of T lymphocyte activation following acute ischemic brain injury in adults has been shown by several studies. Yilmaz et al. were able to show, that Rag (Recombination activating gene) 1−/− mice lacking both T and B lymphocytes had largely reduced infarct size and better neurological outcome than wild type (WT) mice after cerebral ischemia. However, when splenocytes from WT mice were transferred to Rag1−/− mice, the neural damage became comparable in the two groups, indicating that the lymphocytes determining the extent of cerebral damage after HI injury migrate from the periphery to the brain (257, 235). T and B lymphocytes have been shown to appear in the ischemic brain lesion within hours (323, 324) and can be detected for months after HI in adults (268). After perinatal hypoxic-ischemic brain injury, the influx of lymphocytes into the brain is less profound, probably due to the immaturity

of the immune system (260, 325). Therefore, lymphocytes were thought to be of minimal impact on the course of perinatal neuroinflammation, however, recent years' research results brought their role in regulating and maintaining the neuroinflammatory response into light.

Winerdal et al. aimed to characterize the recruitment and activation of T-lymphocytes to the site of the injury following a hypoxic-ischemic insult in a mouse model of perinatal hypoxic brain injury. The link between the innate and adaptive immune system are the antigen presenting cells (APCs) of the brain, which were the activated microglial cells 24 hours after HI. One week later however, a second peak of APC activation was observed, and the majority of these cells were bone-marrow derived APCs, which probably play a central role in sustaining the inflammatory cascade. Increased MHC-II expression and the expression of T cell co-stimulatory CD86 markers was found in APCs already 24 hours after the insult and this could still be observed 3 months later. Similar signs of activation were found in the periphery as well, indicating the presence of massive systemic effects alongside the localized immune response (255). These results indicate, that the large-scale priming of the adaptive immune system is present after perinatal hypoxic brain injury (268).

In line with these findings, the infarcted brain area contained a substantial amount of CD3+ T lymphocytes two weeks after the HI injury, which were not present in the unaffected areas at all. By assessing the temporal alterations of T lymphocytes via flow cytometry, Winerdal et al. were able to demonstrate, that there is a substantial influx of CD4+ T-helper lymphocytes in the acute phase, which peaks one week after HI, and that the recruitment of cytotoxic CD8+ lymphocytes follows a week later and peaks after 2 weeks. The T cells which initially entered the CNS expressed naïve T-cell marker CD45rb, which was downregulated by 3 months. At the same time, T cells began to express activation markers such as CD69 and CD25, which were upregulated even 3 months after the injury, indicating the local activation and maturation of T cells by time. In addition, normally non-immunogenic brain homogenate was able to elicit proliferation in peripheral splenocytes months after HI, which is most likely due to the persisting prevalence of brain-antigen specific activated of T lymphocytes. Authors suggested the possibility, that the long-term adverse neurodevelopmental consequences of HIE might also be related to autoreactive chronic inflammatory

processes (268).

Albertsson et al. were the first who were able to describe the response of the different T helper subsets to HI in the brain and they found, that HI primarily elicited Th1/Th17-type immune response in the neonatal mice (326). They analyzed the expression of the characteristic cytokines and transcription factors of each Th subsets in the brain after HI injury with quantitative polymerase chain reaction (qPCR). They found that Th1-related (T-bet = T-box expressed in T cells, IL-12a) and Th17-related (RORγt = RAR-related orphan receptor C, IL-6, IL-23, and IL-22) genes were significantly upregulated 6-24 h after HI. IL-17A was however not detectable in the brain of neonatal mice. On the contrary the expression of Th2/Treg-related genes were either down-regulated (IL-4) or unchanged (GATA3, IL-5, Foxp3, TGFβ, and IL-10) in the acute phase following HI. This indicates, that HI elicits a robust immune response in the neonatal brain, which is characterized by an imbalance between pro-inflammatory Th1/Th17- and anti-inflammatory Th2/Treg-type responses (326). Authors also suggested, that this imbalance in the type 1 and type 2 cytokine imbalance might be initiated early on, already by innate immune cells. Th1/Th17 predominance has been described in several neuroinflammatory conditions, such as periventricular leukomalacia (PVL) (327-329) and CNS inflammatory demyelination disease (330), and the inhibition of Th17-related cytokines reduced the extent of cerebral damage in a neonatal mouse model of HI (331). Furthermore, Th1 primed activated T-cells were also identified in peripheral blood after stroke (332), indicating, that the investigation of peripheral T cells subsets after HI is reasonable and important.

Several studies support the hypothesis, that T lymphocytes play a pivotal role in maintaining the chronic immuno-inflammatory activation after perinatal HI. Some studies suggest, that adequate adaptive immune response is initiated after 7 days in mouse models, while others show a very early T cell response, however, all studies were able to show the presence and the activation of T lymphocytes in damaged areas until for at least a month after the initial injury (326, 333, 260, 257, 268). Effector T cells are able to exert neurotoxicity via several mechanisms such as the production of perforin and granzyme B, the release of free radicals, the triggering of apoptotic pathways within neurons, and most importantly, the production of pro- and anti-inflammatory cytokines (334, 335).

If T cells are depleted in the periphery by anti-CD3 or sphingosine-1-phosphate (S1P) receptor agonist, ischemia-induced neurodegeneration is ameliorated in adults. On the contrary, in neonates, peripheral T cell depletion leads to the exacerbation of hypoxic-ischemic brain injury. The reason behind this intriguing finding might be, that neonatal HI induces a more pronounced infiltration of Foxp3 positive regulatory T cells, and the inhibition of this process is most likely what leads to the detrimental consequences. However, T cell depletion lead to an increased infiltration of innate immune cells such as neutrophils and inflammatory macrophages, which could also aggravate the neuronal damage (336). Liesz et al. aimed to characterize the role of CD4+CD25+ Foxp3+ Treg lymphocytes in hypoxic brain injury and found, that Treg depletion aggravated delayed brain damage and worsened functional outcome. In the absence of Treg cells, the activation of resident and invading inflammatory cells such as microglia and Foxp3-, effector T cells increased, along with levels of pro-inflammatory cytokines such as TNF-α and IFN-γ. Substituting IL-10 suppressed the cytokine overexpression and prevented secondary infarct growth, indicating the essential role of IL-10 in the immunomodulatory effect of Treg (337).

These data suggest, that certain neonatal T cell subsets also have important immunoregulatory functions after a hypoxic brain injury, which could provide new possibilities of therapeutic intervention. Altogether, even though the relevance of T cells in the development of perinatal hypoxic brain injury is becoming more and more clear, much less data is available on their functional alterations than on those of innate immune cells and human in vivo data is especially scarce. Such results could provide new insights into the course of neuroinflammation in neonates and reveal factors determining neurological outcome.

2.4.3 ADHESION MOLECULES

During neuroinflammation, adhesion molecules are key regulators of leukocyte recruitment and migration to the ischemic lesion. Cell adhesion molecules fall into three large groups of molecules: selectins, the immunoglobulin superfamily and integrins (338). Adhesion molecules present important therapeutic targets and several antibodies are already successfully used in clinical settings (257).

One especially interesting adhesion molecule is the integrin very late antigen

(VLA)-4 (α4/β1, CD49d/CD29), which is responsible for the high-affinity binding of activated leukocytes and activated endothelial cells expressing vascular cell adhesion molecule (VCAM) -1 (339). CD49d is part of the VLA-4 antigen, which in the case of neuroinflammation, mediates the migration of activated lymphocytes through the BBB to the ischemic lesion (340, 341). Antibodies against CD49d have been successfully used in multiple sclerosis to decrease disability progression and the rate of relapses (342), by inhibiting lymphocyte trafficking into the brain. The blocking of VLA-4 by antibodies was also shown to be protective 72 h after HI in adults and in murine ischemic stroke models (343, 232). Although VCAM-1 is generally expressed by inflamed endothelial cells and is not specific for the CNS, after a hypoxic brain insult, it will primarily be expressed in the vessels near the ischemic lesion. At the same time, the level of CD49d expression on T cells can be correlated with their capacity to enter the CNS (344). Even though they appear to be promising targets for modulating the T lymphocyte trafficking into the brain, the alterations of adhesion molecules in HIE are not well understood and experimental data is lacking.

2.4.4 CYTOKINES

The complex network of cytokines has been shown to play a crucial role in mediating the neuroinflammatory response triggered by HI and determining the extent of brain injury (345-347, 212, 348, 349). During neuroinflammation the primary source of cytokines are glial cells and immune cells, such as T helper cells (161). Cytokines are often categorized based on their effect as pro- or anti-inflammatory. Pro-inflammatory cytokines maintain the ongoing inflammation by inducing further cytokine release and continuous leukocyte migration, leading to the accumulation of neurotoxic agents (350). Pro-inflammatory cytokines have been implicated as mediators of both the early events and the latent, long-term neuroinflammatory response (161). TNF-α and IFN-γ have also been shown to be directly neurotoxic in vitro (347). Together with IL-1β, they appear to play a central role in the development of perinatal hypoxic brain injury (351-353), although IL-6, IL-8 and IL-17 are also likely to have an important contribution (354-357). On the other hand, anti-inflammatory cytokines such as IL-4, IL-10, IL-11, IL-13 and TGFβ ameliorate the inflammation and TGF-β and IL-10 have been shown to be neuroprotective and crucial

for regenerative processes (1).

However, the dual role of neuroinflammation following HI can already be recognized at level of the cytokine network, as apparently opposing effects of classical cytokines have been uncovered during the past decade. This might be explained by the fact, that cytokines, chemokines, and growth factors are ubiquitous signaling molecules and there is a significant overlap between the CNS and the immune system in the expression of these molecules (347). In fact, T cells and other immune signals have been shown to play a role in adult hippocampal neurogenesis and spatial learning (358, 359). It also appears, that while excess amount of pro-inflammatory cytokines can aggravate neuronal injury, a certain level is essential for the fast elimination of cellular debris, neuronal growth and repair, and thus functional recovery (360, 302). The results of Saliba et al. also support the positive role of pro-inflammatory cytokines (i.e. TNF-α and IL-1) in neuronal regeneration (355). The effect and role of individual cytokines in perinatal hypoxic brain injury has been the highlight of the past decades' research and the following pages will summarize some of these results.

2.4.5 PRO-INFLAMMATORY CYTOKINES

2.4.5.1 IL-1β

IL-1β is a member of the IL-1 protein family and is an important mediator of inflammatory responses, cell proliferation, differentiation, and apoptosis. IL-1β is also one of the most important pro-inflammatory cytokines that have been associated with poor neurological outcome after HI injury (161). Many animal studies have demonstrated its role in both the early and the late stages of hypoxia-induced neuroinflammation (361-368). Upon hypoxic insult, IL-1β mRNA and protein levels are rapidly upregulated in the ipsilateral hemisphere of the neonatal brain and they remain elevated during the course of the neuroinflammatory response (369, 370, 297, 371, 372). Continuously elevated IL-1β levels have been associated with permanent neuronal damage (373, 372). Although it appears, that IL-1β is not directly toxic to oligodendrocyte lineage cells, it does inhibit oligodendrocyte proliferation, which can delay white matter development and recovery (374). One of the main effects of IL-1β is the transcriptional activation of the iNOS gene (375) leading to NO generation (376, 377), which is known to contribute to BBB breakdown and apoptotic neuronal death

(378, 368). Exogenous administration of IL-1β leads to the exacerbation of ischemic brain injury. Further supporting the relevance of IL-1β, it has been demonstrated that both the deficiency of the IL-1β converting enzyme and treatment with IL-1 receptor antagonists (IL-1ra) or blocking antibodies results in the moderation of the hypoxic brain injury (370, 379), decrease in the cellular edema (368), and improved neurological outcome (380). The beneficial effects of IL-1β down-regulation may be due to decreased microglial activation, leukocyte infiltration, and lower cytokine levels (370, 381, 382).

There is however also some in vitro evidence, suggesting that IL-1β could have neurotrophic effects, exerted via the stimulation of nerve growth factor synthesis. Direct intracerebral injection of IL-1 or TNF-α have been shown to induce astrogliosis and angiogenesis in the developing rodent brain (383).

The primary sources of IL-1β are APCs and monocytes (384, 385), although microglia and endothelial cells are also capable of producing IL-1β (386-388). However, the fact that T lymphocytes are able to produce physiologically relevant amounts of IL-1β and that it plays an important role in their functionality has only been recently revealed. Doitsh et al. were the first to describe functional IL-1β production in CD4 T lymphocytes in HIV infection (389), and Arbore et al. were able to demonstrate, that while APC-derived IL-1β supports initial T cell priming (390), proper imprinting of T helper effector function and maintenance of the Th1 phenotype during differentiation and migration into the periphery appears to rely on autocrine T-cell derived IL-1β production (391). They also revealed, that the dysregulation of autocrine IL-1β mediated pathway leads to Th1-Th17 imbalance (389). Mice with deletion of IL-1β signal transducer are also unable to form memory T cells (392). Altogether, this indicates, that contrary to previous beliefs, T lymphocytes are capable of IL-1β production, in fact, it plays an indispensable role in the maintenance of their function.

2.4.5.2 IL-6

The understanding of the effects of IL-6 has changed over the past decade, as evidence emerged of its role in both pro- and anti-inflammatory processes. IL-6 has been demonstrated to exert both detrimental and neurotrophic effects in cerebral

ischemia, depending on the pathologic context (347). During neuroinflammation, IL-6 is produced by astrocytes, possibly microglia and migrating immune cells such as monocytes and T lymphocytes (393). It has been suggested based on rodent models of HI, that IL-6 contributes to the exacerbation of the inflammatory response in the acute phase of hypoxic brain injury, and more to the neuronal regeneration in the secondary and tertiary phases (394). In line with this, the cerebral level of IL-6 shows a transient increase, and peaks hours after hypoxic injury, which has been associated with enhanced neuronal damage (370, 395). On the other hand, astrocyte-derived IL-6 has been shown to enhance neuronal survival and protect from NMDA receptor-mediated excitotoxicity after hypoxia-reoxygenation challenge in vitro (396, 397). The neuroprotective and neurotrophic effects of IL-6 might be mediated via leukemia inhibitory factor and ciliary neurotrophic factor (398, 399, 397, 161).

2.4.5.3 IL-17

IL-17 is a pro-inflammatory cytokine often associated with detrimental consequences in inflammatory diseases. It is primarily produced by a subset of T lymphocytes upon IL-23 stimulation, called Th17 cells (400), although other cells types, such as CD8+ T cells are also capable of producing IL-17 (401). IL-17 also appears to be involved in the late stages of neuroinflammation, playing a role in the delayed progression of brain infarction. In an experimental ischemic stroke model, the size of the infarcted area was significantly reduced in IL-17KO mice from the 4th post-stroke day onwards, accompanied by decreased apoptotic neuronal death (402).

It appears, that Th17 response could also be an important link between systemic inflammation and aggravated hypoxic-ischemic CNS injury. IL-23R and IL-17 levels were higher both in rat pups challenged by LPS-sensitization and infants with a history of chorioamnionitis who sustained HI brain injury. The blocking of lymphocyte trafficking to the CNS by sphingosine-1-phosphate receptor (S1PR) agonist resulted in decreased CNS CD4+ and IL-17+ cell count and rescued up to 90% of LPS/HI-induced brain tissue loss in a dose-dependent manner in mice. However, it did not affect tissue loss due to “pure” HI injury, highlighting its efficacy in inflammation-sensitized HI injury. This indicates, that Th17-mediated immunity could play a critical role in the development of perinatal hypoxic brain injury following exposure to

inflammation (284, 403).

2.4.5.4 IFN-γ

IFN-γ is another classical Th1-type, pro-inflammatory cytokine which has been demonstrated to prompt neuronal development. IFN-γ has also been suggested to play a dual role in HI brain injury. Data indicates, that depending on the timing and level of expression, IFN-γ could have destructive and protective effects in the brain (161). IFN-γ is an important mediator of Th1-type cellular immune responses and is produced in large amounts by Th1 cells, although reactive microglial cells, macrophages and astrocytes are also important sources in the early stages of neuroinflammation. IFN-γ drives further Th1 differentiation and the production of pro-inflammatory cytokines, such as TNF-α (404). Its molecular effects include ROS production and lipid peroxidation, which add to its neurotoxicity (328). However, the contribution of IFN-γ to neuronal survival, differentiation, ramification, and myelin formation has also been demonstrated. Experimental data even suggests, that IFN-γ could act as a neuroprotective agent by decreasing the apoptosis of oligodendrocyte progenitors (405) and astrocytes (406) in rodent models of HI. Elevated levels of IFN-γ were observed in the cord blood of neonates with white matter injury in the first 72 hours of life (328, 407). However, the role and contribution of IFN-γ to the development of perinatal HI brain injury is not clear and experimental data is lacking (161).

2.4.5.5 TNF-α

The data published, that supports the role of TNF-α in the development of ischemic brain damage is surmounting (408, 370, 409, 410, 395). TNF-α has also been suggested to play a role in perinatal stroke, however further studies are necessary to assess its role. TNF-α is a classical pro-inflammatory cytokine, that stimulates the production of IL-1β and other cytokines, promotes leukocyte differentiation, proliferation and CNS infiltration (411, 412). On the other hand, TNF-α also plays a role in neuronal progenitor cell proliferation, lineage commitment and cellular differentiation. Direct intracerebral injection of IL-1 or TNF-α has been shown to stimulate astrogliosis and angiogenesis in the developing rodent brain (284, 383).

TNF-α binds to two different receptors, that activate distinct signaling pathways. On the one hand, excess amounts of TNF-α may induce apoptosis via TNF-R1 (413), on the other hand binding to TNF-R2 can prompt cell survival, growth and proliferation (414). Exposure to TNF-α reduced oligodendrocyte progenitor survival and maturation in vitro and led to reduced myelination (415, 405, 161). TNF-α induced apoptosis in both developing and mature oligodendrocytes (416, 417). In vivo, after HI insult, oligodendrocytes expressed higher levels of TNF-R1, which could explain the higher rate of apoptosis and the delayed demyelination (362, 418). TNF-α expression began to increase early and peaked 6-12 hours following hypoxic ischemic injury in newborn rats, indicating its role in the early events of neuroinflammation (419). Higher TNF-α and IL-1β plasma and CSF levels in term neonates with perinatal HIE have been associated with worse neurological status at 12 months of age and higher incidence of cerebral palsy (420, 421). Upon inhibition of TNF-α by pentoxifylline, a competitive inhibitor, neurological outcome improved. Blocking TNF-α resulted in reduced BBB disruption and also protected neurons from delayed cell death in animal models of head trauma (422).

2.4.6 ANTI-INFLAMMATORY CYTOKINES

2.4.6.1 IL-10

IL-10 is a classic anti-inflammatory cytokine, mostly produced by Tregs and activated monocytes and macrophages. Its anti-inflammatory effects are exerted by the down-regulation of the activation and cytokine production of microglia and macrophages (406). The cerebral level of IL-10 shows a significant increase after HI (369, 161). In vitro data shows, that IL-10 is able to exert neuroprotective effects decreasing hypoxia-induced neuronal death and LPS- or IFN-induced oligodendrocyte loss (423-425). IL-10 has also been shown to neutralize the metabolic and microcirculatory effects of perinatal HI in piglets (426).

However, in vivo a neuroprotective effect could only be observed, if IL-10 was administered exogenously following HI. Neither IL-10 blocking antibodies or IL-10 KO had any effect on the size of the brain lesion, indicating that endogenous IL-10 appears to have a lesser role in moderating the neuroinflammatory response. IL-10

administration was however able to counteract the deleterious effects of pro-inflammatory cytokines IL-1β and IL-9, suggesting, that exogenous IL-10 can exert neuroprotective effects in an inflammatory context (406). In one clinical observational study, preterm neonates who were found homozygous for the high IL-10 producer -1082 G-allele were significantly less likely to develop periventricular lesions (427).

2.4.6.2 TGF-β

The TGF-β family consists of pleiotropic proteins with potent immunoregulatory properties, which are also important regulators of the development, repair and survival of neurons (355). The most important immunosuppressive effects of TGF-β are the direct inhibition of inflammatory cells and the promotion of Treg function by inducing their Foxp3 expression (428-434). Activated Tregs then produce large amounts of TGF-β, which maintains the immunosuppressive milieu (435). TGF-β specifically limits Th1 differentiation and expansion (436, 437) without altering Th2 effector function. It also suppresses the production of pro-inflammatory cytokines, while promoting the production of anti-inflammatory IL-10 (438). In addition to direct inhibition, Tregs also inhibit T cell function by interfering with the APC - T cell interactions, for example by activating the cytotoxic T lymphocyte antigen - 4 (CTLA-4) pathway, which induces tryptophan (TRP) catabolism by indoleamine 2,3-dioxygenase (IDO) (284, 439, 440).

An increasing body of evidence supports the neuroprotective effect of TGF-β in HI brain injury (441). The induction of TGF-β1 synthesis reduced the size of the infarcted area in rats (442). Exogenously administered TGF-β was also effective in reducing infarct volume in several animal models (443-445). One study showed, that direct cerebral administration of TGF-β improved short-term neurological function after HI injury adult rats, however, it did not have a long-term neuroprotective effect (347, 446). One clinically especially relevant finding is that non-invasive, intranasal TGF-β administration was also effective in reducing the affected area and improving functional recovery in mice (447). On the other hand, TGF-β -inhibition lead to exacerbated HI brain injury, demonstrating the neuroprotective value of endogenous TGF-β production (441, 448).

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2.4.7 THE KYNURENINE SYSTEM

The interplay between the cytokine network and the kynurenine system regulates both innate and adaptive immune responses, and is also important in the bidirectional relationship of the central nervous system and the immune system (449, 450). IDO is an inducible enzyme, which catalyzes the first, rate-limiting step of TRP catabolism. It is a key mediator of neuroimmune interactions. IDO degrades TRP to kynurenine (KYN), which is then metabolized by the enzymes of the kynurenine pathway into further catabolites, such as kynurenic acid (KYNA). Certain TRP metabolites can exert neurotoxic properties, however, the primary metabolite, KYNA has been shown to have neuroprotective effects. In vitro KYNA ameliorates NMDA receptor-mediated excitotoxicity in the human neocortex (451), exhibits high free radical scavenging activity and is an endogenous inhibitor of oxidative stress (452).

IDO is mainly produced by APCs and is a potent immunosuppressive agent. It is induced by pro-inflammatory signals (such as IFN-γ), and its most important function is the maintenance of the immunobalance. Tryptophan depletion renders effector T cells inactive and APCs immunosuppressive (453). IDO induction and the activation of the kynurenine pathway also leads to the activation of regulatory T cells and the inhibition of natural killer cells. The rate of TRP degradation can be measured by the ratio of KYN to TRP (K/T), which allows close estimation of the enzymatic activity of IDO (454). The alterations of the kynurenine system appear to be important in the pathophysiology of a broad spectrum of neurological disorders (455), however its role in perinatal hypoxic-ischemic cerebral injury has not been investigated yet (284).

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3 STUDY RATIONALE

The neuroinflammatory response, which is a common consequence of HI brain injury, appears to have two sides. A certain level of inflammation is part of the physiological response to any injury and is essential in reparative processes of the infarcted tissue (282). However, there is substantial evidence demonstrating the detrimental consequences of excessive neuroinflammation, leading to exacerbated CNS injury and worse neurological outcome.

In this study one of our primary focuses was to determine which aspects of the neuroinflammatory response could differ between neonates with moderate HIE, who are likely to have normal neurodevelopmental outcome or mild disability and neonates with severe HIE who are likely to have poor outcome (death or severe disability). We focused on characterizing the components of the adaptive immune system, more specifically T lymphocytes, since there is little in vivo data available regarding the alterations of T cells during perinatal HI brain injury.

The majority of previous studies performed on samples from neonates with HIE focused on determining the plasma cytokine levels, which raises many concerns. Plasma cytokine levels are secreted by a wide variety of innate and adaptive immune cells, and it is therefore difficult to comment on their specific local role in the CNS. As previously described, cytokines are capable of exerting even opposing effects depending on the milieu. Furthermore, the diffusion of cytokines from the plasma to the inflammatory focus (in HI injury the CNS) is marginal compared to the local production. It is possible however, that specific immune cells which are active at the inflammatory focus are present in the peripheral circulation. Therefore, by characterizing specific cellular subsets it is possible to gain a more precise understanding of events during an inflammatory process. Another consideration is, that plasma cytokine levels show larger variability and are less stable over time, than intracellular cytokines, which remain within the observed cells until the time of measurement and thus more closely reflect the cytokine production at a cellular level (456, 457). Due to these reasons we primarily focused on the intracellular cytokine production of T lymphocytes, however we also measured plasma cytokine levels to try to form a more comprehensive overview of the immunological alterations following perinatal HI injury.

Due to the high level of ubiquity in the cytokine network, it is hard to identify a single factor, which determines long-term outcome, even more so, because the effect of cytokines is highly dependent on the context (type of insult, timing) (347). However, certain cytokine profiles have been associated with detrimental consequences (161), and current research is focused on identifying these key inflammatory signatures. The real goal would be to distinguish between the level of inflammation necessary for CNS regeneration and the characteristics of inflammation which lead to poor outcome.

There is also emerging evidence indicating, that perinatal HI injury could be followed by an extended period of chronic neuroinflammation, which could alter many aspects of neurodevelopment and contribute to the long-term consequences of perinatal HI injury. Although there are emerging studies on the long-term effects of neuroinflammation on neurodevelopment, the majority of these data are circumstantial, and little is known about the events of this phase of the neuroinflammatory response in humans. Therefore, we extended our observation period to the whole first month of life to try to gain a better understanding of the tertiary, chronic phase of the neuroinflammatory process. Such data from human samples have not been published previously. Gaining a better understanding of the long-term effects of perinatal HI brain injury could open novel opportunities for targeted interventions.

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4 AIMS

In this study our aims were the following:

1. To assess the differences in the prevalence and cytokine production of T lymphocyte subsets between moderate and severe HIE, in order to identify the players of the inflammatory response that may influence the severity of the neuroinflammation
2. To assess the alterations of plasma cytokine levels in comparison with intracellular cytokine levels in moderate and severe HIE
3. To describe the plasma levels of the substances of the kynurenine system (TRP, KYN and KYNA) and assess IDO activity based on the KYN/TRP ratio in moderate and severe HIE
4. Based on the pooled data collected in the first month of life from four NAIS patients, we aimed to assess the gross differences in the cytokine production of T lymphocytes and plasma cytokine levels between moderate, severe HIE and NAIS. Similar data has not been published in humans before, therefore, although the number of NAIS cases is small, the presented data could serve as a base for future, larger-scale case-control studies.

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5 MATERIALS AND METHODS

5.1 PATIENTS

We enrolled 33 term neonates in our study, all of whom were outborn and admitted to the regional neonatal intensive care unit at the First Department of Pediatrics at Semmelweis University, Budapest, Hungary with the initial diagnosis of perinatal asphyxia. The diagnosis of moderate-to-severe hypoxic-ischemic encephalopathy and the eligibility for cooling were assessed according to the TOBY criteria (88). Neonates, who had congenital abnormalities, CNS malformations or were born from mothers who had signs of chorioamnionitis were not included in the study. All enrolled neonates met the criteria for therapeutic total body hypothermia, which was initiated upon admission, between 1-5 hours of life. During the 72-hour hypothermia period, the rectal temperature of neonates was recorded every hour and maintained between 33-34 °C. Clinical characteristics and laboratory parameters of participants are summarized in Table 4.

Study-related blood sampling was adjusted to clinical care, 2x1 ml of peripheral venous blood was collected together with blood samples necessary for clinical care. Samples were collected on 5 occasions from each neonate: between 3-6 hours of life (at admission), at 24 hours, at 72 hours and at 1 week of life during the intensive care treatment and at 1 month of age during a routine outpatient follow-up appointment. Samples were processed within 6 hours in all cases, samples were not cooled during this period.

Upon admission blood cultures and ear swabs were obtained from all neonates to exclude perinatal bacterial infection. All neonates received prophylactic intravenous antibiotic treatment (ampicillin-gentamycin) in the first days of life, which were stopped after negative blood cultures were received. Infants were monitored for infection during the first month of life, clinical or culture-proven sepsis was not detected in any participants. Neonates received standard intensive care, during the study period 13 neonates required inotropic therapy and 10 neonates required hydrocortisone as additional blood pressure supportive therapy, 3 neonates required L-thyroxin-supplementation due to low fT4 serum levels, 12 neonates received red-blood-cell concentrate on at least one occasion due to anemia, 3 neonates received

platelet concentrate due to low platelet count (< 100G/l) and 16 infants received at least one dose of fresh-frozen-plasma due to coagulopathy.

Neonates were monitored by aEEG and MRI examinations were performed within the first week of life if possible, and within 12 days of life in all cases. MRI data were interpreted by radiologists who were blinded to the clinical status of the neonates, based on criteria defined by Rutherford et al. (458, 30). Based on the MRI scan results, four neonates were diagnosed with neonatal arterial ischemic stroke. These cases are presented in detail below. One neonate was excluded from the study due to metabolic disease (peroxisomal fatty acid C26/C22 ratio was above the normal range) along with the presence of multiple minor anomalies and the mutation of the ROBO1 gene. The other 28 neonates were divided into two groups (moderate and severe HIE) depending on the severity of hypoxic-ischemic encephalopathy, which was determined based on the initial status and the time of normalization of the aEEG recordings (459) and the MRI scan results. A structured MRI reporting template was developed by a collaborating research group in the ISORT (intelligent structured online reporting tool) software framework created by Bioscreen Ltd., Debrecen, Hungary based on the work of Marcovici et al. and Bosmans et al. (460, 461).

The severe group (n = 11) was constituted of neonates who:

- **A.** Had signs of moderate-to-severe HIE on the MRI scans AND
- **B.** Met at least one of the following criteria:
    - I. Had signs of moderate-to-severe HIE on the aEEG, defined as:
        - i. burst-suppression
        - ii. continuous extremely low voltage background activity
        - iii. flat tracing background activity
    - II. The normalization of the aEEG activity occurred after 48 hours of life or never
    - III. Early death occurred (< 28 days).

The moderate group (n = 17) was constituted of neonates, who

- **A.** Met none of the above listed criteria, AND
- **B.** Had normal MRI scans or mild signs of HIE on the MRI scans, AND
- **C.** Their aEEG recordings met at least one of the following criteria:
  - I. Normal voltage background activity
  - II. Normalization of aEEG activity occurred before 48 hours of life.

Table 4. Clinical characteristics of included neonates. The initial laboratory results are presented, which were collected upon admission (within 12 h of age).

*Data are presented as median (IQR). Data were compared with Mann-Whitney tests. *p < 0.05 vs Moderate HIE, ** p < 0.05 vs severe HIE.*

	Moderate HIE (n = 17)	**Severe HIE (n = 11)**	**NAIS (n = 4)**
Clinical characteristics			
Male gender (%)	10 (59%)	7 (64%)	2 (50%)
Birthweight (g)	3330 [2860-3605]	3000 [2490-3300]	3115 [2540-3885]
Gestational age (wk)	39 [37-40]	38 [37-40]	39,5 [38.25-40]
No. of C-sections (%)	10 (59%)	8 (73%)	3 (75%)
Apgar at 1 min	3 [0.5-4.5]	1 [0-3]	4 [3-5]
Apgar at 5 min	6** [5-7]	2 [0-4]	5,5** [5-6]
Apgar at 10 min	7** [5-8]	4 [1.75-5.25]	7 [6-7]
Worst pH	7.025 [6.87-7.12]	6.86 [6.62-7.06]	6.94 [6.88-7.00]
Worst BD (mmol/L)	18.05 [16.65-21.28]	20.4 [19.38-23.5]	14** [14-17]
S100 (µg/L)	7.48 [2.33-28.85]	21.8 [3.8-30.0]	3.60 [1.88-18.35]
LDH (U/L)	2072 [1371-5274]	3335 [1879-5792]	1874 [1676-3187]

In the severe group, 3 infants deceased before one month of age due to the severity of the HI insult. In these cases, the MRI examination could not be performed due to the critical condition or early death of the patient, therefore these cases were included in the severe group based on the aEEG results and the poor putcome. Data, which were available from these neonates were included at respective time points. Overall, 72 hour, 1 week and 1 month data were missing from 2 infants and only 1 month data were missing from 1 infant. Pooled data from the cohort of 4 NAIS patients were compared to neonates with HIE.

Our study was reviewed and approved by the Hungarian Medical Research Council (TUKEB 6578-0/2011-EKU) and written informed consent was obtained from parents of all participants. The study was adhered to the tenets of the most recent revision of the Declaration of Helsinki. The data and protocols presented here have been published (233, 284).

5.2 NAIS CASE PRESENTATIONS

5.2.1 CASE 1.

The neonate was delivered by an emergency caesarean section due to oligohydramnios and fetal tachycardia on the 40th week of gestation, following an uncomplicated pregnancy. The mother had no history of chronic illnesses, no signs of maternal chorioamnionitis were present, however it should be noted, that the mother smoked during her pregnancy. The amniotic fluid was stained with thick meconium, and the Apgar score was 5/6/7. The neonate had to be resuscitated after delivery and required intubation and assisted ventilation in the first days of life. The first capillary blood gas showed deep acidosis (pH 7.03, BE -14 mmol/L, lactate 12.4 mmol/L) and she soon became irritable, her general muscle tone increased, thus, hypothermic treatment was initiated. On the second day of life she presented with symptoms characteristic of pulmonary hypertension, which resolved after one day of NO inhalation and she was extubated on Day 3 of life. Her early neurodevelopmental examination showed mild central hypotonia.

Cranial MRI with diffusion-weighted imaging was performed on Day 4, and showed a distinct, 5x7 mm ischemic area with decreased diffusion in the left thalamus

and no evidence of bleeding. MR spectroscopy showed a decrease in metabolites, characteristic of slight general hypoxia-ischemia.

5.2.2 CASE 2.

The neonate was delivered by an emergency caesarean section due to complete placental abruption on the 39th week of gestation with an Apgar score of 3/5/8. The mother had no history of any illnesses. At birth no respiratory effort was present, and the neonate was hypotonic and pale, but had a heartrate of 80/min. After short bag and mask ventilation, his heartrate normalized, but only gasping was present, thus he was intubated. The neonate soon began to show signs of neurological involvement, such as irritability. The first blood gas showed severe metabolic acidosis (pH 6.99, BE -14), therefore hypothermic treatment was initiated. A chest X-ray was performed due to ventilation difficulty, which showed a pneumothorax on the right side, which was drained. The neonate required blood transfusion on two occasions due to severe anemia and low blood pressure, and inotropic support for six days. An early neurodevelopmental examination indicated grossly abnormal central and peripheral tone distribution.

Cranial MRI was performed on Day 4 of life. Diffusion-weighted imaging showed a large area of ischemia with decreased diffusion in the area of the left middle cerebral artery, and several smaller lesions in the area of the right middle cerebral artery. No signs of bleeding were present. Time-of-flight (TOF) MR angiography showed marked irregularity and significantly decreased blood flow in both MCAs, but especially on the left side. No signs of hypoxic-ischemic encephalopathy were present on the MRI scan.

5.2.3 CASE 3.

The neonate was born by a normal vaginal delivery on the 40th week of gestation, following an uncomplicated pregnancy with an Apgar score of 5/5. The neonate was hypotonic, and spontaneous breathing was not initiated after birth, therefore she was intubated. The first gasping breaths were observed at 15 minutes after birth, but the neonate remained hypotonic. The first blood gas showed severe acidosis (pH 6.89, BE -18 mmol/L, lactate 15 mmol/L). Therapeutic hypothermia was

initiated. The initial aEEG recordings showed abnormal background activity, the normalization occurred within a few hours. The early neurodevelopmental examination reported moderate generalized hypotonia.

The cranial MRI examination was performed on Day 3 of life. Diffusion-weighted imaging showed a small (3 mm) ischemic lesion in the right thalamus. Further ischemic areas, bleeding or signs of hypoxic ischemic encephalopathy were not present.

5.2.4 CASE 4.

The neonate was born by emergency caesarean section following an uncomplicated pregnancy on the 38th week of gestation due to fetal distress. His Apgar score was 3/6/7, he was flaccid with no spontaneous breathing and a heart rate of 80/min. After bag and mask ventilation spontaneous breathing was noted at 5 minutes, but the neonate remained hypotensive and areflexive, therefore he was intubated. The initial blood gas showed severe acidosis (pH 6.875, BE -14). Therapeutic hypothermia was initiated. The initial aEEG recording showed no signs of seizures. The early neurodevelopmental examination indicated abnormal central and peripheral tone distribution.

Cranial MRI was performed early, on the first day of life. Diffusion-weighted imaging showed decreased diffusion indicating ischemia in the area of the right posterior cerebral artery. Smaller lesions were present in the right thalamus and the left parieto-occipital area. No signs of bleeding or hypoxic ischemic encephalopathy could be observed.

The data presented here have been published (233).

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5.3 SUMMARY OF MEASURED PARAMETERS

Table 5. Overview of all measured parameters and the methods used.

For flow cytometry, all measurements were done on whole blood samples where red blood cells were lysed. Intracellular cytokine levels were evaluated within each subset, identified by labelling characteristic cell-surface markers. Immunoassays and High-performance liquid chromatography (HPLC) measurements were performed from plasma samples.

	Associated cell subsets	Place of measurement	Method
Cell surface markers			
CD4	T helper lymphocytes (Th)	Cell surface	Flow cytometry
CD8	Cytotoxic T lymphocytes (CTL)	Cell surface	Flow cytometry
CD49d	Extravasation marker (i. e. to the CNS)	Cell surface	Flow cytometry
Pro-inflammatory factors			
IL-1β	Macrophages, smaller amount produced by T cells	Intracellular Plasma	Flow cytometry Immunoassay
IL-2	Main T cell activation factor, high amounts by Th1	Plasma	Immunoassay
IL-6	Th17 cells, also exerts anti-inflammatory properties	Intracellular Plasma	Flow cytometry Immunoassay
IL-7	Stromal cells, dendritic cells, for lymphocyte development	Plasma	Immunoassay
IL-8	Macrophages, known as neutrophil chemotactic factor	Plasma	Immunoassay
IL-12	Th1 cells (differentiation signal)	Plasma	Immunoassay
IL-17	Th17 cells (CD4+) and Tc17 cells (CD8+)	Intracellular Plasma	Flow cytometry Immunoassay
IFN-γ	Th1 cells, smaller amounts in CTL	Intracellular Plasma	Flow cytometry Immunoassay

G-CSF	Endothelium, macrophages, neutrophil maturation	Plasma	Immunoassay
MCP-1	Monocyte chemoattractant protein (chemokine)	Plasma	Immunoassay
MIP-1β	Macrophage inflammatory protein (chemokine)	Plasma	Immunoassay
TNF-α	Macrophages, Th lymphocytes	Intracellular	Flow cytometry
		Plasma	Immunoassay
VCAM	Vascular adhesion of immune cells to endothelium	Plasma	Immunoassay
Anti-inflammatory factors			
IL-4	Th2 cells (differentiation signal)	Plasma	Immunoassay
IL-5	Th2 cells	Plasma	Immunoassay
IL-10	Treg cells	Intracellular	Flow cytometry
		Plasma	Immunoassay
IL-13	Th2 cells	Plasma	Immunoassay
Foxp3	Key transcription factor of Treg cells	Intracellular	Flow cytometry
GM-CSF	Treg, both pro- and anti-inflammatory effects	Plasma	Immunoassay
TGF-β	Treg	Intracellular	Flow cytometry
		Plasma	Immunoassay
KYN	Ubiquitous	Plasma	HPLC
KYNA	Ubiquitous	Plasma	HPLC
TRP	Ubiquitous	Plasma	HPLC

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5.4 FLOW CYTOMETRY

The 2 ml-s of peripheral blood samples were centrifuged to separate plasma, plasma samples were aliquoted and immediately frozen at -80 °C for later determination of plasma cytokine concentrations and HPLC measurements (see below). An overview of all measured parameters are provided in Table 5.

The remaining fraction was resuspended in RPMI (Roswell Park Memorial Institute-1640 medium, Sigma-Aldrich, St. Louis, MO, USA) to 2 ml end volume. Samples were incubated with PMA (Phorbol 12-myristate 13-acetate) (50 ng/ml), ionomycin (1 microg/ml) and BFA (Brefeldin A) (10 microg/ml) for 6 h at 37 °C to allow intracellular accumulation of cytokines.

After 6 hours, samples were washed, divided into four equal aliquots:

- P1+: for labeling of the cytokines of Panel 1
- P1-: for the isotype controls of cytokines in Panel 1
- P2+: for labeling of the cytokines of Panel 2
- P2-: for the isotype controls of cytokines in Panel 2

Cell surface markers were labeled with the following fluorochrome-conjugated anti-human monoclonal antibodies according to the manufacturers' instructions: CD4 PE-Cy7 (Phycoerythrin-Cyanine 7) and CD8 APC-Cy7 (Allophycocyanin-Cyanine 7) in Panel 1, or CD4 APC-Cy7 and CD49d PerCP (Peridinin-Chlorophyll-Protein) in P2+ tube and only CD4 APC-Cy7 in P2- tube (all from BioLegend, San Diego, CA, USA). Tubes were incubated for 30 minutes at room temperature.

Red blood cells were then lysed and PBMCs were permeabilized using FACSLysing and FACSPermeabilizing solutions according to manufacturer's instructions (BD Biosciences, San Jose, CA, USA). 1 ml of 1x FACS Lysing solution was added to each tube, after gentle vortexing, tubes were incubated for 20 minutes at room temperature. Cells were then washed, centrifuged and resuspended in 500 µl of 1x FACS Permeabilizing solution. Tubes were incubated for 10 minutes at room temperature.

Cells were washed, centrifuged and resuspended in PBS (phosphate buffer saline) for intracellular staining using isotype controls. The following conjugated anti-human monoclonal antibodies or the appropriate isotype controls were applied

according to the manufacturers' instruction: IL-6 PE (Phycoerythrin), IL-17A PerCP (Peridinin Chlorophyll Protein Complex), IL-10 APC (Allophycocyanin), IFN-γ FITC (Fluorescein Isothiocyanate) (for Panel 1), or TNF-α PE-Cy7, Foxp3 PE, TGF-β APC, IL-1β FITC (for Panel 2), all from BioLegend. Samples were incubated for 30 minutes at room temperature.

After intracellular labeling, cells were washed and resuspended in 300 µl of PBS for analysis by flow cytometry. Samples were measured immediately on a FACSAria flow cytometer (BD Biosciences) equipped with 488 and 633 nanometer excitation lasers and FACSDiVa software (BD Biosciences). 100,000 cells were recorded. Gating and analysis was performed using FlowJo, LLC (284).

5.5 IMMUNOASSAYS

Plasma samples were stored at -80 °C until analysis. The plasma levels of the following cytokines, chemokines and growth factors were measured using Bio-Plex Pro Assays (Bio-Rad Laboratories, Hercules, CA, USA): IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, IFN-γ, TNF-α, TGF-β, G-CSF, GM-CSF, MCP-1, MIP-1β and VCAM. Bio-Plex Pro Assays are immunoassays similar to a sandwich ELISA. Capture antibodies against the biomarker of interest are covalently coupled to magnetic beads. A biotinylated detection antibody creates the sandwich complex and the final detection complex is formed by the addition of a streptavidin-phycoerythrin (SA-PE) conjugate, where PE serves as the fluorescent reporter. Reactions are read using a Luminex-based reader (284).

5.6 HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Plasma samples were stored at -80 °C until analysis. Directly prior to analysis, samples were thawed, vortexed and 300 µl of plasma was "shot" onto 700 µl of precipitation solvent (containing 3.57 w/w% perchloric acid and 2.857 mM 3-nitro-L-tyrosine as internal standard (Scharlau, Barcelona, Spain)). Samples were then centrifuged (13000 G for 10 minutes at 4 °C) and the supernatants were collected. For the quantification of KYN, KYNA, and TRP concentrations of samples, a modified method was used

based on the work of Herve et al. (462), using an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA). The system was equipped with a fluorescent detector, by which the concentration of KYNA and TRP was determined, and a UV detector, by which the concentration of KYN and the internal standard was determined. Chromatographic separations were performed on an Onyx Monolithic C18 column, 100 mm × 4.6 mm I.D. (Phenomenex Inc., Torrance, CA, USA) after passage through a Hypersil ODS precolumn, 20 × 2.1 mm I.D., 5 µm particle size (Agilent Technologies) with a mobile phase composition of 0.2 M zinc acetate/acetonitrile 95/5 v/v% with a pH adjusted to 6.2 with glacial acetic acid, applying isocratic elution. The flow rate and the injection volume were 1.5 ml/minute and 20 µl, respectively. The fluorescent detector was set at excitation and emission wavelengths of 344 nm and 398 nm respectively, and after 3.5 minutes of each run, the wavelengths were changed to 254 nm and 398 nm. The UV detector was set at a wavelength of 365 nm. L-TRP, L-KYN sulfate salt, KYNA and zinc acetate dihydrate were purchased from Sigma-Aldrich and acetic acid was purchased from VWR International (Radnar, PA, USA) (284).

5.7 STATISTICAL ANALYSIS

First, test of normality was performed (according to Kolmogorov-Smirnoff), which indicated non-normal distribution of data. Therefore, Mann-Whitney tests were used to make comparisons between two different sample populations. For comparisons between the paired values (samples collected at different time points from the same patients) within the same population, Friedman test was used. Outliers were identified using Grubbs' tests and were excluded from analyses. Data are expressed as median and interquartile range, p values less than 0.05 were considered significant. For statistical analysis and calculations, the GraphPad Prism 5 software (La Jolla, CA, USA) was used.

6 RESULTS

We found significant alterations in both the prevalence of T lymphocytes expressing certain cytokines, the intracellular level of cytokines and the plasma levels of cytokines between our study groups. The main focus of our study, the differences between moderate and severe HIE will be presented first. Differences between NAIS and HIE groups will be shown later. Pro- and anti-inflammatory factors will be presented separately.

To create a systematic way of presenting our data, the following method of showing significant differences is used in all tables and figures. To present differences between two study populations, stars are used: * if the data is different compared to moderate HIE at the same time point, and ** if the data is different compared to severe HIE at the same time point. To present differences between different time points within the same study population letters are used in the following manner: “a” if the difference is significant compared to the 6 h value, “b” if the difference is significant compared to the 24 h value, “c” if the difference is significant compared to the 72 h value and “d” if the difference is significant compared to the 1 wk value.

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6.1 COMPARISONS BETWEEN THE MODERATE AND THE SEVERE HIE GROUPS

6.1.1 PRO-INFLAMMATORY CYTOKINES

Table 6. Differences in pro-inflammatory cytokines between moderate and severe HIE groups. *The prevalence of T cells expressing certain cytokines are shown as the percentage of the parent population. The intracellular levels of cytokines are shown by the mean fluorescence intensity (MFI) of each cytokine within the subset of T cells in arbitrary unit. Plasma cytokine levels are shown in pg/ml. Data are expressed as median (IQR). Only significant alterations are shown, $p \leq 0.05$ in all cases.*

	Time	Moderate HIE (n = 17)	Severe HIE (n = 11)
Cell prevalence data (% of parent population)			
CD4+ IL-1β+/CD4+	6 h	3.52 (2.13-5.16)	6.77 (3.18-10.26)
CD4+ IL-1β+ CD49d+/ CD4+ IL-1β+	6 h	6.98 (4.61-9.32)	4.08 (2.86-5.46)
CD4+ TNF-α+ CD49d+/ CD4+ TNF-α+	6 h	6.63 (4.47-13.45)	3.52 (2.12-7.23)
CD8+ IL-17+/CD8+	6 h	5.26 (3.89-14.40)	2.30 (1.73-4.49)
CD4+ TNF-α+ CD49d+/ CD4+ TNF-α+	72 h	4.77 (3.43-7.70)	9.75 (6.31-10.80)
CD4+ IL-17+/CD4+	1 wk	3.08 (1.80-4.59)	5.13 (3.40-13.76)
Intracellular cytokine levels - MFI data (arbitrary unit)			
IL-17 / CD8+	24 h	1069 (639-3265)	4187 (1274-6133)
IFN-γ / CD4+	72 h	455 (150-770)	887 (496-1427)
IL-17 / CD4+	72 h	939 (566-1674)	1760 (1614-3508)
TNF-α / CD4+	1 mo	3281 (1752-4326)	4729 (3959-6714)
Plasma cytokines (pg/ml)			
G-CSF	24 h	19.85 (10.87-30.70)	42.74 (22.27-131.3)
G-CSF	1 wk	13.33 (5.52-17.72)	32.90 (16.65-94.76)
IL-6	1 wk	21.06 (11.89-43.24)	70.25 (33.73-134.1)

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6.1.1.1 IL-1β

We found an elevated prevalence of IL-1β-expressing CD4 lymphocytes at 6 h after birth in severe HIE compared to moderate HIE. These cells also showed a higher rate of extravasation to the CNS at 6 h in severe HIE, indicated by the decrease in the prevalence of CD49d-expressing CD4+ IL-1β+ cells in the peripheral blood (Table 6). The prevalence of these cells did not differ at later time points.

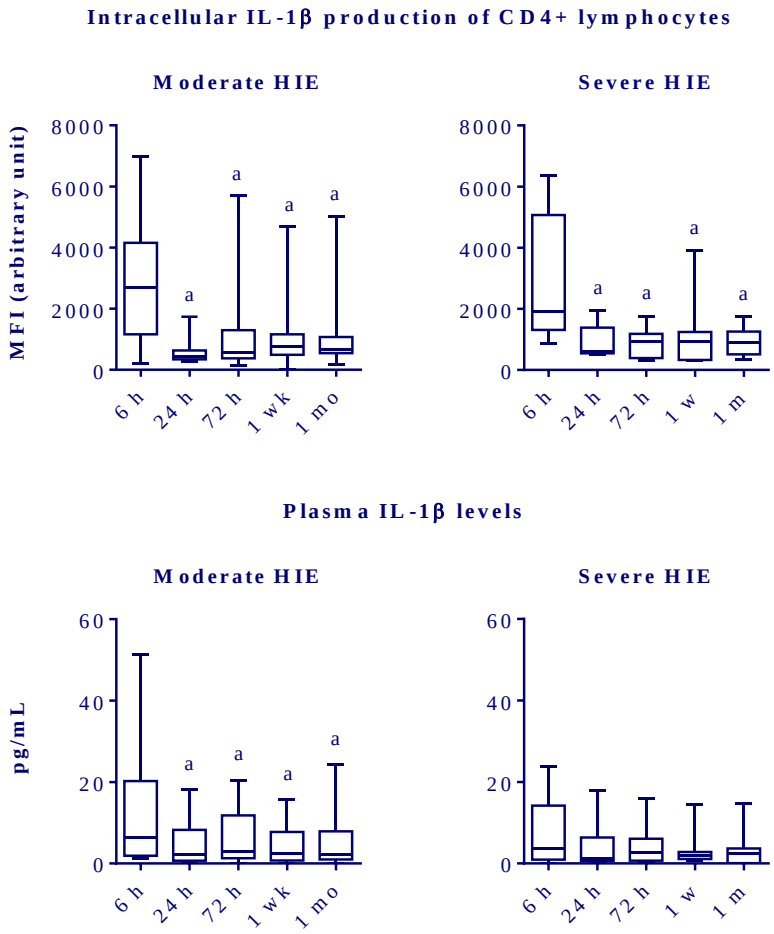

Figure 10. Alterations in the intracellular IL-1β level of CD4+ lymphocytes and plasma IL-1β levels during the first month of life within the moderate and severe HIE. Intracellular level is shown by the MFI of IL-1β. Plasma IL-1β level is shown in pg/ml. Moderate HIE (n = 17), severe HIE (n = 11). Horizontal line: median, box: interquartile range, whisker: range. $p \leq 0.05$ a vs 6 h (all within the same group).

However, the intracellular levels of IL-1β, indicated by the mean fluorescence intensity (MFI) of IL-1β were similar in the two study groups, the peak was at 6 h and intracellular IL-1β levels were comparably lower at the following time points. The plasma levels of IL-1β changed similarly in the moderate HIE, and showed a similar tendency in the severe group, but did not reach the level of significance (Figure 10). No differences were present in the plasma level of IL-1β between moderate and severe HIE. These data support the role of IL-1β in the initiation of the neuroinflammatory response.

6.1.1.2 IL-6

The prevalence of IL-6 producing T lymphocytes and the intracellular IL-6 levels were comparable in the two HIE groups, no significant difference was present. Interestingly, in both of the HIE groups CD4+ lymphocytes expressed the highest level of IL-6 at 24 h, indicated by the peak of the MFI of IL-6 at this time point. IL-6 production decreased afterwards, suggesting that IL-6 may also play an important role in the initial phase of the neuroinflammatory response (Figure 11).

We found a difference in the plasma level of IL-6, which was higher in severe HIE at 1 wk than in moderate HIE. We found a decrease in plasma IL-6 levels in the moderate group by 1 mo, which was not significant in the severe one (Figure 11).

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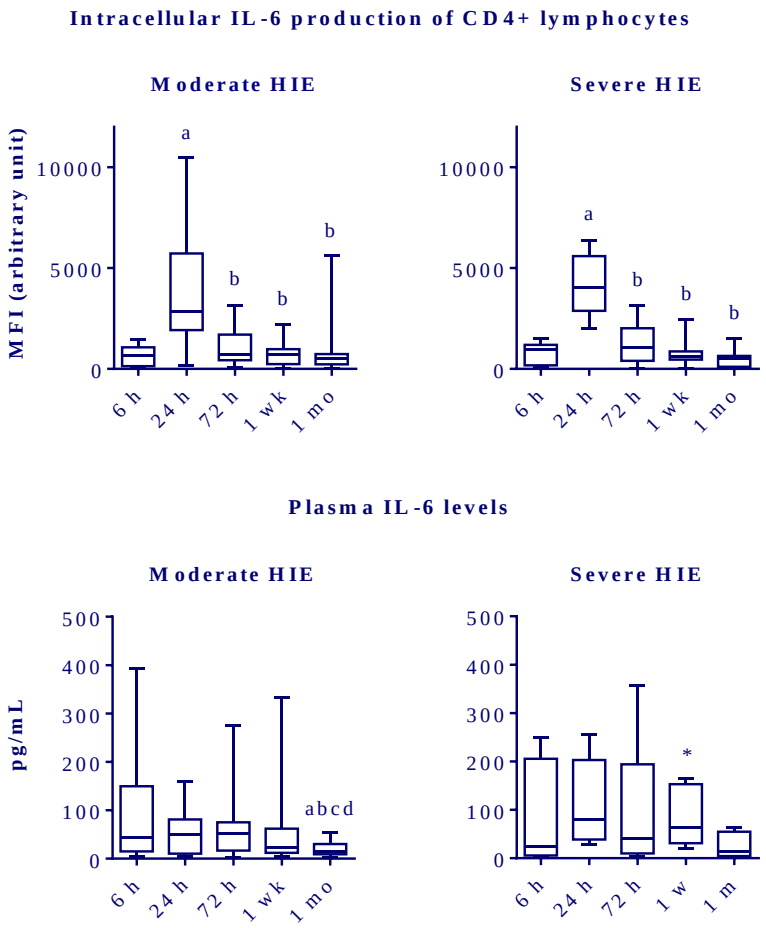

Figure 11. Alterations in the intracellular IL-6 level of CD4+ T cells and plasma IL-6 levels during the first month of life within the moderate and severe HIE groups. *Intracellular level is shown by the MFI of IL-1β. Plasma IL-1β level is shown in pg/ml. Moderate HIE (n = 17), severe HIE (n = 11). Horizontal line: median, box: interquartile range, whisker: range. $p \leq 0.05$ a vs 6 h, b vs 24 h, c vs 72 h, d vs 1 wk (all within the same group), * = vs moderate at the same time point.*

6.1.1.3 IL-17

The prevalence of IL17-producing CD4+ T cells, known as Th17 cells increased by 24 h compared to 6 hours of life and remained elevated during the first month of life. (Figure 12). At 1 wk this value was significantly higher in severe HIE (Table 6). Intracellular IL-17 production (indicated by the MFI of IL-17) was also higher in the severe group compared with the moderate group at 72 h in CD4+ lymphocytes. (Table 6).

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The prevalence of CD8+ IL17+ T lymphocytes was on the other hand higher in moderate, than in severe HIE at 6 h (Table 6). Within the moderate group, IL-17 production (indicated by the MFI of IL-17) peaked at 72 h and decreased by 1 mo in CD8+ cells. However, at 24 h, the intracellular IL-17 level of CD8+ cells was somewhat higher in severe HIE (Figure 12) No differences could be detected between the two study groups in the plasma levels of IL-17.

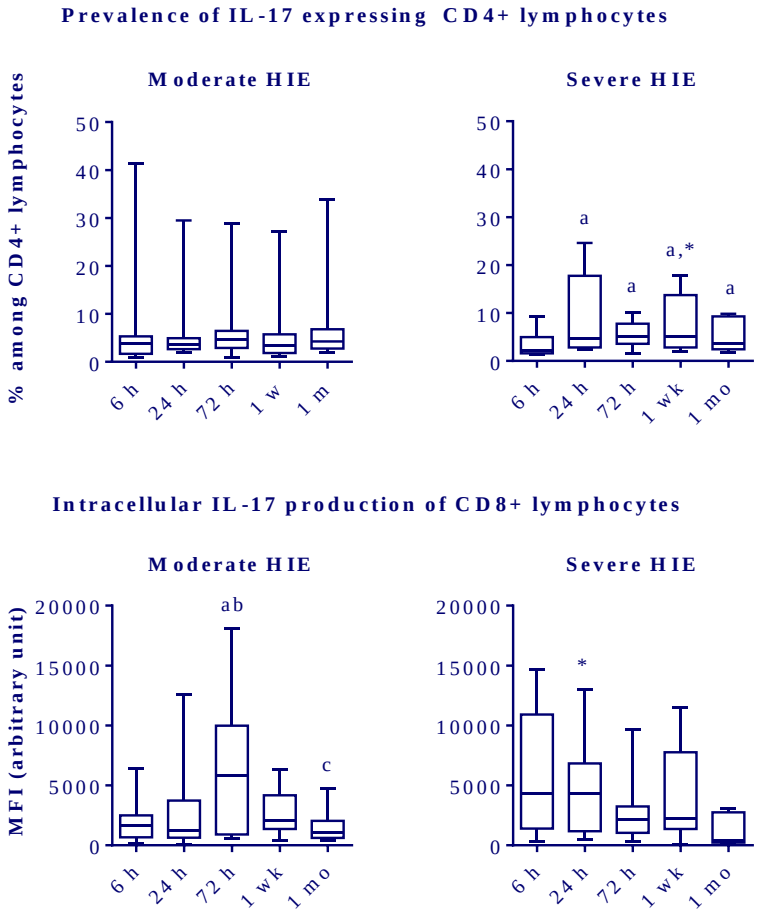

Figure 12. Differences in the prevalence of IL-17 producing CD4+ lymphocytes and intracellular IL-17 production of CD8+ lymphocytes during the first mo of life in moderate and severe HIE. *Cell prevalence data is shown in the percentage of the parent population. Intracellular level is shown by the MFI of IL-1β. Moderate HIE (n = 17), severe HIE (n = 11). Horizontal line: median, box: interquartile range, whisker: range. $p \leq 0.05$ a vs 6 h, b vs 24 h, c vs 72 h (all within the same group), * = vs moderate at the same time point.*

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6.1.1.4 TNF-α

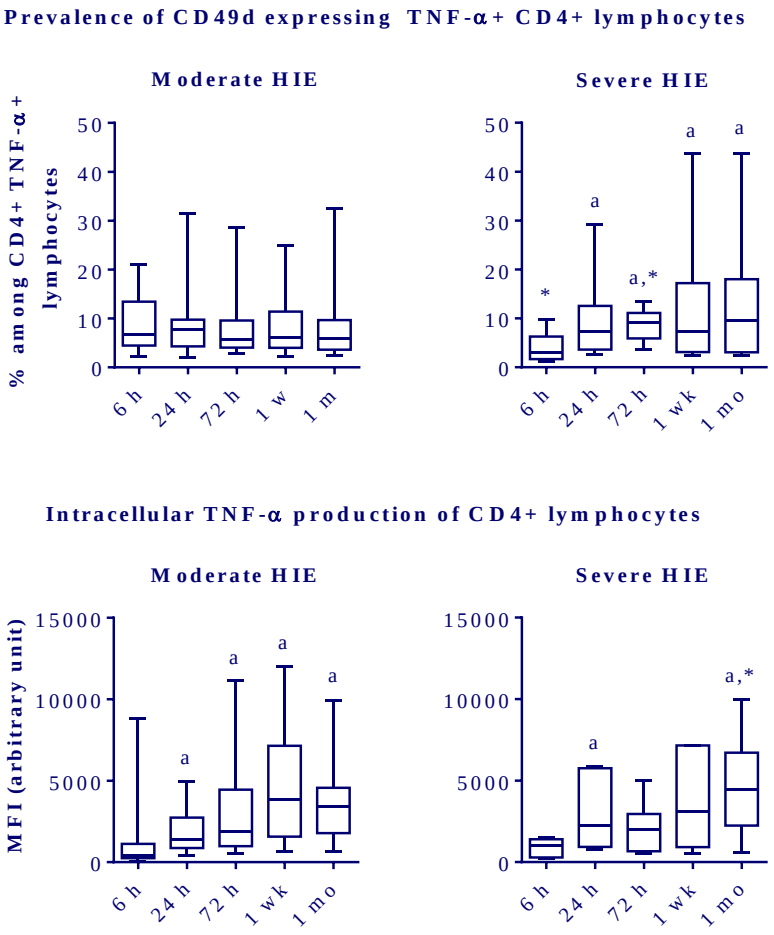

Figure 13. Differences in the prevalence of CD49d producing TNF-α+ CD4+ lymphocytes and intracellular TNF-α production of CD4+ lymphocytes during the first mo of life in moderate and severe HIE. *Cell prevalence data is shown in the percentage of the parent population. Intracellular level is shown by the MFI of IL-1β. Moderate HIE (n = 17), severe HIE (n = 11). Horizontal line: median, box: interquartile range, whisker: range. $p \leq 0.05$ a vs 6 h (within the same group), * = vs moderate at the same time point.*

The TNF-α production of CD4+ lymphocytes (MFI of TNF-α) was increased at all time points compared to 6 h in both HIE groups. By 1 mo, the MFI of TNF-α in CD4+ cells was higher in the severe group than in the moderate, suggesting the presence of an ongoing inflammatory response, which could contribute to the long-term consequences of perinatal asphyxia. Plasma levels of TNF-α did not differ.

The prevalence of CD49d-expressing CD4+ TNF-α+ lymphocytes, which are prone to enter the CNS is lower in severe HIE at 6 h compared to moderate HIE and also compared to later time points, indicating increased extravasation. The prevalence of CD49d-expressing CD4+ TNF-α+ cells then increased and became higher in severe HIE than in moderate HIE by 72, which could be a result of increased CD49d production (Table 6 and Figure 13).

6.1.1.5 OTHER PRO-INFLAMMATORY CYTOKINES

The MFI of IFN-γ in CD4+ cells was elevated in severe HIE compared to moderate HIE at 72 h. Plasma G-CSF levels were higher in the severe group than in the moderate group at 24 h and 1 wk (Table 6).In moderate HIE, plasma G-CSF levels showed a decrease by 1 wk and remained low during the rest of the first month of life. Plasma MCP-1 levels within the moderate group were elevated at 24 h, 72 h and 1 wk compared to 6 h (Figure 15) (284).

6.1.2 ANTI-INFLAMMATORY FACTORS

Table 7. Differences in anti-inflammatory factors between moderate and severe HIE groups. *The prevalence of T cells expressing certain cytokines are shown as the percentage of the parent population. Plasma cytokine levels are shown in pg/ml. The concentration of the factors measured by HPLC is shown in μM. Data are expressed as median and interquartile range. Only significant alterations are shown, $p \leq 0.05$ in all cases.*

	Time	Moderate HIE (n = 17)	Severe HIE (n = 11)
Cell prevalence data (% of parent population)			
CD4+ Foxp3+/CD4+	24 h	2.35 (1.96-3.13) %	3.02 (2.60-4.13) %
Plasma cytokines (pg/ml)			
IL-5	72 h	1.37 (0.00-4.69)	0.20 (0.00-0.46)
IL-13	72 h	2.35 (2.01-3.67)	1.70 (1.40-2.56)
HPLC results (μM)			
KYN	1 mo	3.62 (2.72-4.47)	2.28 (1.45-3.14)

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6.1.2.1 TGF-β

In the moderate group, the prevalence of CD49d-expressing CD4+ TGF-β+ cells increased after 72 h and remained elevated until the end of the first month of life. This indicates a greater potential for TGF-β producing cells to enter the CNS in from 1 wk onwards moderate HIE. CD4+ cells also expressed higher levels of TGF-β from 24 h onwards in the moderate group. Although similar tendencies were present in the severe group alterations did not reach significant levels (Figure 14).

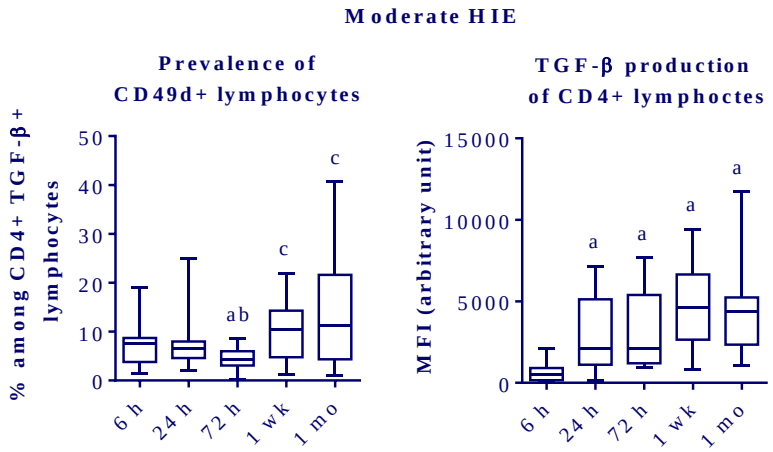

Figure 14. Differences in the prevalence of CD49d producing TGF-β+ CD4+ lymphocytes and intracellular TGF-β production of CD4+ lymphocytes during the first month of life in moderate HIE. *Cell prevalence data is shown in the percentage of the parent population. Intracellular level is shown by the MFI of IL-1β. Moderate HIE (n = 17). Horizontal line: median, box: interquartile range, whisker: range. p ≤ 0.05 a vs 6 h, b vs 24 h, c vs 72 h (all within the same group).*

6.1.2.2 TREGS

The prevalence of Tregs was somewhat higher in severe HIE at 24 h, which might be a result of a compensatory mechanism, however, the biological significance of this increase is difficult to determine (Table 7) (284).

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6.1.2.3 OTHER ANTI-INFLAMMATORY CYTOKINES

In the moderate group, plasma IL-10 levels decreased by 1 mo and were lower than at 6 and 24 h. In the severe group, plasma IL-13 levels decreased by 72 h and were lower than at 6 and 24 h in the following timepoints (Figure 15). Plasma IL-13 and IL-5 levels were higher in the moderate than in the severe group at 72 h (Table 7).

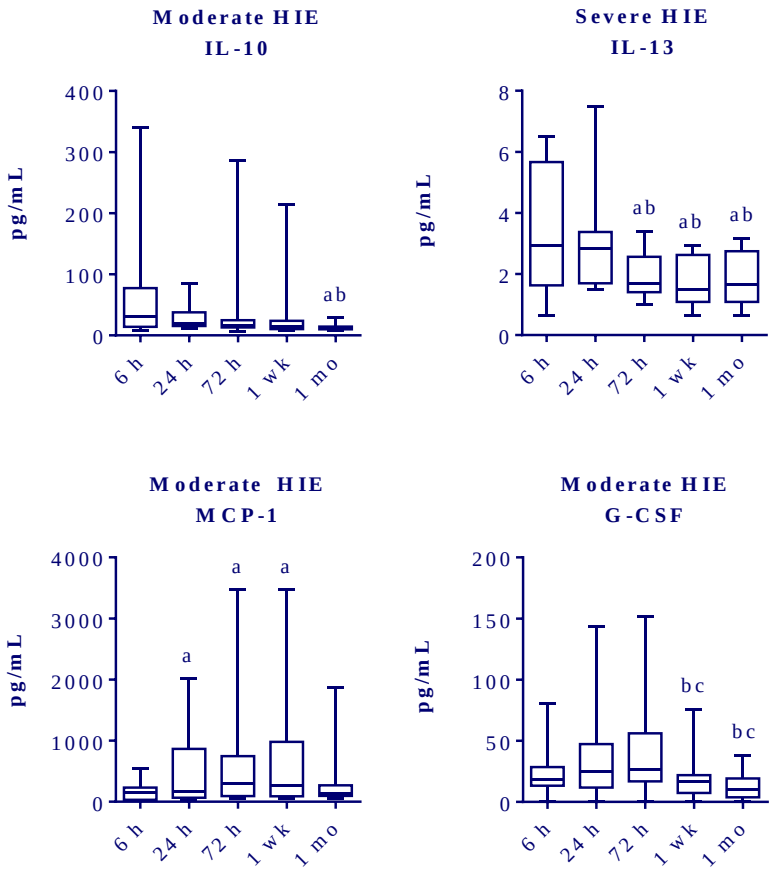

Figure 15. Alterations of the plasma levels of anti-inflammatory cytokines in moderate and severe HIE. *Plasma cytokine levels are shown in pg/mL. Moderate HIE (n = 17), severe HIE (n=11). Horizontal line: median, box: interquartile range, whisker: range. $p \leq 0.05$ a vs 6 h, b vs 24 h, c vs 72 h (all within the same group).*

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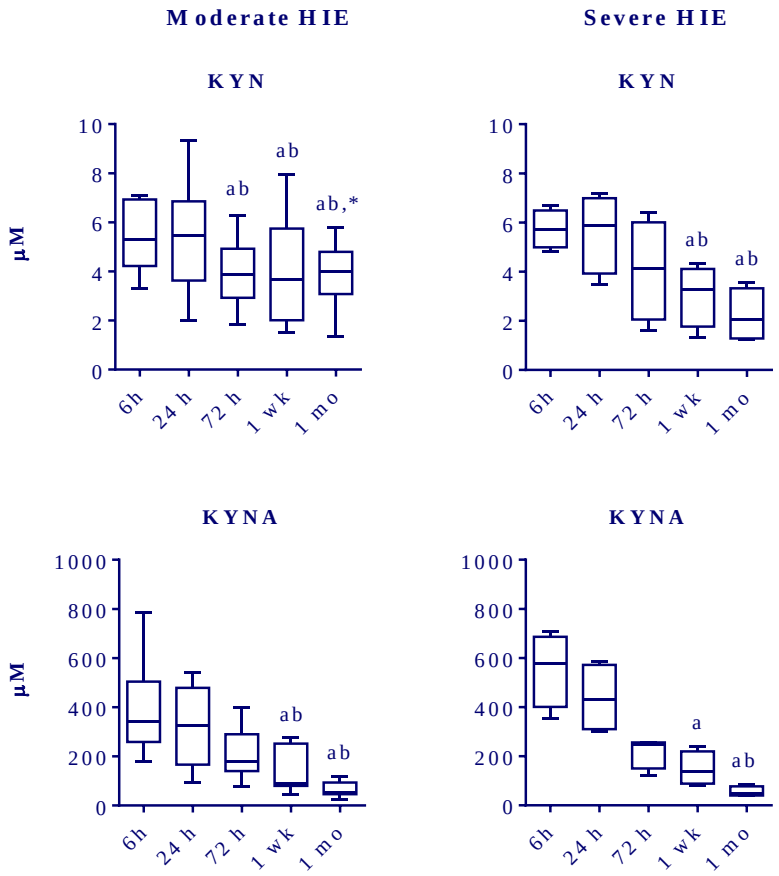
6.1.2.4 THE KYNURENINE SYSTEM

The plasma levels of KYN were higher in moderate HIE at 1 mo than in severe HIE (Table 7). The components of the kynurenine system otherwise showed similar alterations in time within the two HIE groups. TRP levels showed a gradual increase in both groups by the end of the first month of life. Parallelly, plasma KYN and KYNA levels showed a gradual decline until 1 mo. In line with the above alterations, the enzymatic activity of IDO, indicated by the ratio of KYN and TRP (K/T ratio) also showed a decline by the end of the first month after the perinatal HI injury (Figure 16).



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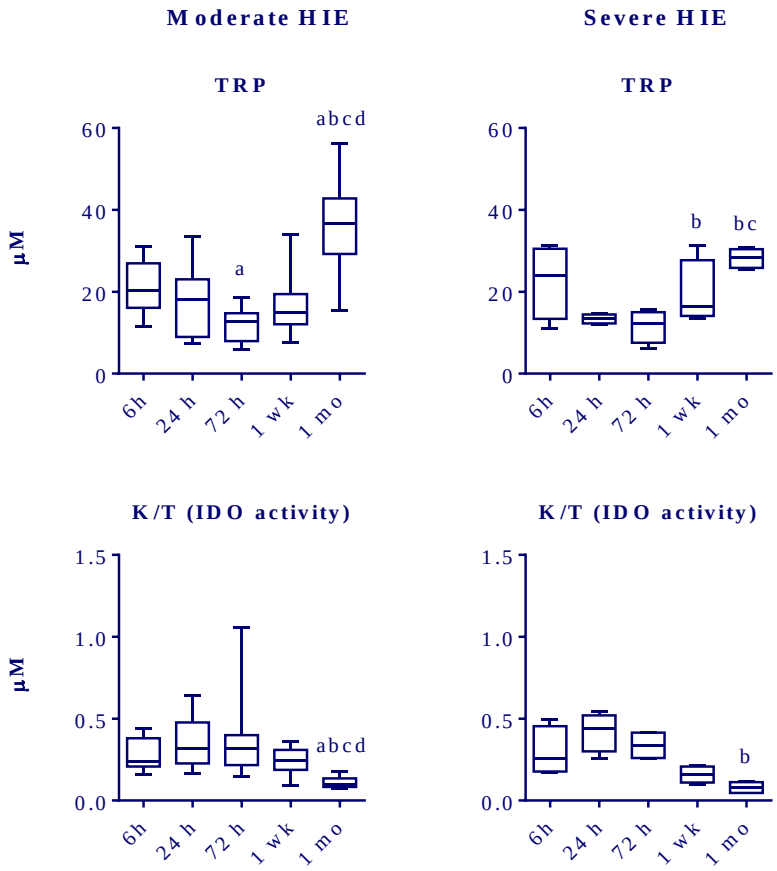

Figure 16. Alterations in the components of the kynurenine pathway in time in moderate and severe HIE. *Values are shown in µM. Horizontal line: median, box: interquartile range, whisker: range. KYN – kynurenine, KYNA – kynurenic acid, TRP – tryptophan, K/T – kynurenine/tryptophan ratio. p ≤ 0.05 a vs 6 h, b vs 24 h, c vs 72 h, d vs 1 wk (all within the same group). * = vs moderate at the same time point.*

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6.1.3 ROC ANALYSIS

We also assessed each parameter's value in distinguishing between moderate and severe HIE by ROC analysis. We found, that all significant results are connected to IL-1β. By assessing the prevalence of IL-1β-producing CD4+ T cells at 6 h ($p = 0.018$, ROC AUC = 0.784) and the CD49d expression of the above cells at 6 h ($p = 0.027$, ROC AUC = 0.767) we could differentiate between moderate and severe insult with a reasonable sensitivity and specificity (Figure 17) early on (284).

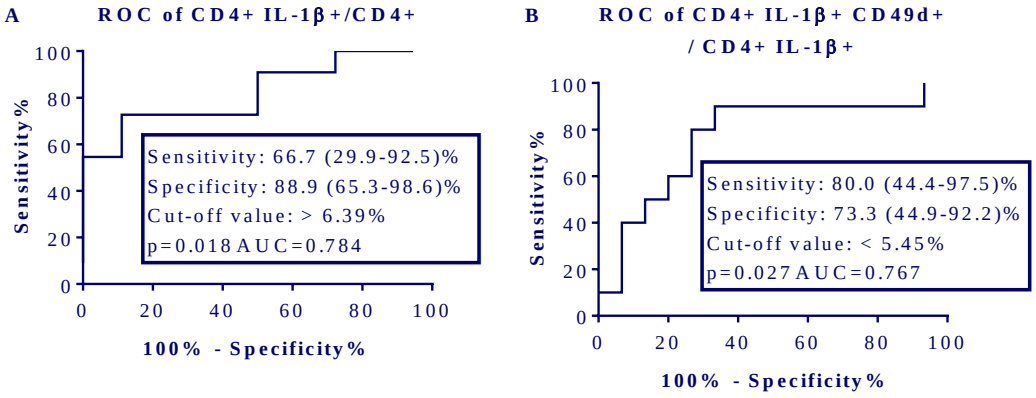

Figure 17. Receiver operator curve (ROC) analysis to differentiate between moderate and severe HIE based on the prevalence of A) CD4+ IL-1β+ T cells and B) the ratio of CD49d+ within the CD4+ IL-1β+ T cell subset. *AUC – area under the curve.*

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6.2 COMPARISONS BETWEEN HIE AND NAIS

6.2.1 CYTOKINE PRODUCTION OF T LYMPHOCYTES

Table 8. Differences in the intracellular levels of pro-inflammatory cytokines and the prevalence of T cells expressing pro-inflammatory cytokines between HIE and NAIS. *The prevalence of T cells expressing certain cytokines is shown as the percentage of the parent population. The intracellular levels of each cytokine are shown by the mean fluorescence intensity (MFI) of each cytokine in arbitrary unit. Data are expressed as median and interquartile range. We are showing the study populations between which significant differences are present in bold. p≤ 0.05 * = vs moderate, ** = vs severe.*

	Time	Moderate HIE (n = 17)	Severe HIE (n = 11)	NAIS (n = 4)
Cell prevalence data (% of parent population)				
CD8+ IL-17+ / CD8+	6 h	**5.26 (3.89 -14.40) **, p ≤ 0.05**	**2.30 (1.73-4.49)**	**5.04 (4.62-7.68) **, p ≤ 0.05**
CD4+ IFN-γ+ / CD4+	72 h	6.40 (3.70-11.80)	6.17 (4.75 -13.01)	17.50 (2.74-30.20)
Intracellular cytokine levels - MFI data (arbitrary unit)				
IL-17 / CD8+	6 h	**1427 (661.5-2402)**	1591 (1157-7047)	**4855 (2618-8706) *, p ≤ 0.01**
IL-6 /CD8+	72 h	701,0 (364.8-1441)	**1073 (795.0-2017)**	**646.5 (188.7-736.3) **, p ≤ 0.05**
IFN-γ / CD4+	72 h	**455.0 (149.5-770.0)**	**887.0 (495.5-1427)**	**42.25 (28.13-438.5) *, p ≤ 0.05 **, p ≤ 0.05**
IL-6 / CD8+	1 mo	**429,0 (101.7-617.0)**	**347.0 (83.00-491.0)**	**918.5 (638.3-1347) *, p ≤ 0.05 **, p ≤ 0.05**

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6.2.1.1 PRO-INFLAMMATORY CYTOKINES

Similar to our observations in HIE, CD4+ T lymphocytes produced the highest level of IL-1β at 6 hours in NAIS as well. This was indicted by the elevated MFI value of IL-1β, which decreased significantly by 24 hours. We found no difference between NAIS and HIE groups in the intracellular production of IL-1β (Figure 18).

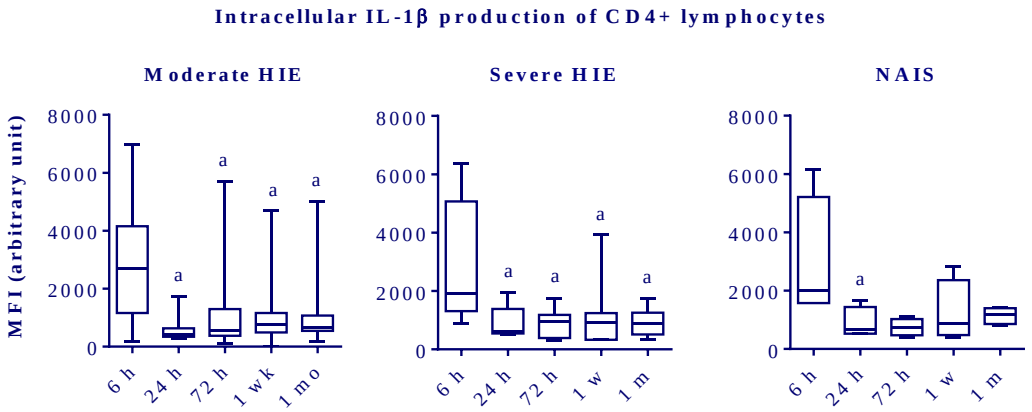

Figure 18. Alterations in the intracellular IL-1β level of CD4+ lymphocytes, during the first mo of life in moderate, severe HIE and NAIS. *Intracellular level is shown by the MFI of IL-1β. Moderate HIE (n = 17), severe HIE (n = 11), NAIS (n = 4). Horizontal line: median, box: interquartile range, whisker: range. $p \leq 0.05$ a vs 6 h (within the same group).*

The MFI of IL-6 in CD8+ cells at 72 h was lower in NAIS than in severe HIE. However, by 1 mo the MFI of IL-6 in CD8+ cells decreased in both HIE groups but became higher in NAIS (Table 8).

At 6 h the prevalence of CD8+ lymphocytes producing IL-17 was higher in NAIS than in severe HIE and CD8 cells also expressed higher levels of IL-17 at 6 h in NAIS than moderate HIE (Table 8).

In NAIS, CD4+ lymphocytes expressed the highest level of IFN-γ at 24 h. Intracellular IFN-γ level decreased significantly by 72 h, by when it became lower in NAIS than in either HIE groups. The prevalence of IFN-γ+ CD4 cells did not differ between the study populations (Table 8 and Figure 19).

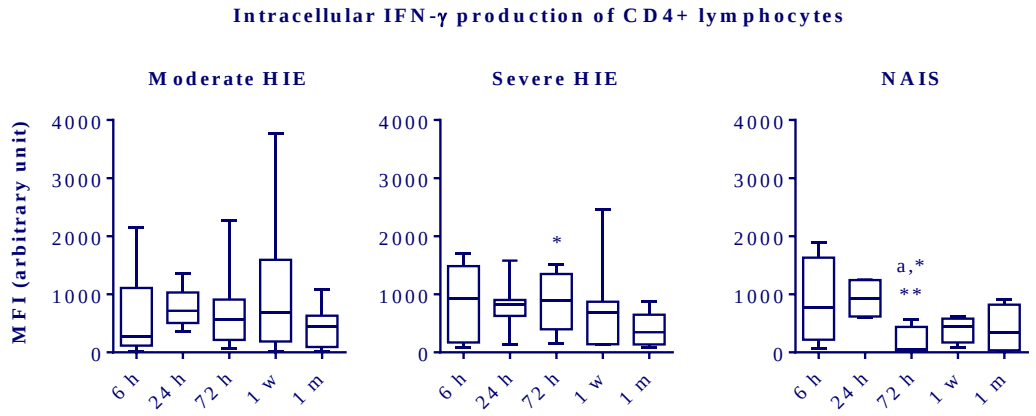

Figure 19. Alterations in the intracellular IFN-γ level of CD4+ lymphocytes, during the first month of life in moderate, severe HIE and NAIS. *Intracellular level is shown by the MFI of IFN-γ. Moderate HIE (n = 17), severe HIE (n = 11), NAIS (n = 4). Horizontal line: median, box: interquartile range, whisker: range. p ≤ 0.05 a vs 6 h, b vs 24 h, c vs 72 h (all within the same group), * = vs moderate at the same time point, ** = vs severe at the same time point.*

6.2.1.2 ANTI-INFLAMMATORY CYTOKINES

The prevalence of IL-10+ CD8 lymphocytes was lower in NAIS than in the severe HIE at 6 h and 72. Although the prevalence of IL-10+ CD8 lymphocytes remained consistently lower in NAIS, the difference was not significant level at the 24 h time point. On the other hand, the prevalence of IL-10+ CD4 cells was higher at 24 h in NAIS than in moderate HIE (Table 9) (233).

Table 9. Prevalence of T lymphocytes producing anti-inflammatory cytokines
*The prevalence of T cells expressing anti-inflammatory cytokines are shown as the percentage of the parent population. Data are expressed as median and interquartile range; $p \leq 0.05$ * = vs moderate HIE, ** = vs severe HIE. Data, where significant differences were present compared to NAIS are shown in bold.*

		Moderate HIE (n = 17)	**Severe HIE (n = 11)**	**NAIS (n = 4)**
Cell prevalence data (% of parent population)				
CD8+ IL-10+ / CD8+	6 h	2.75 (1.19-4.12)	**4.19 (1.94-6.91)**	**1.10 (0.40-1.36) **, $p \leq 0.01$**
CD4+ IL-10+ / CD4+	24 h	**2.88 (1.56-7.03)**	4.52 (3.02-6.59)	**9.47 (5.10-20.68) *, $p \leq 0.05$**
CD8+ IL-10+ / CD8+	72 h	3.51 (1.85-5.12)	**5.30 (2.41-6.08)**	**1.39 (0.52-2.99) **, $p \leq 0.05$**
CD4+ TGF-β+ CD49d+ / CD4+ TGF-β+	1w	**10.54 (4.77-14.28)**	**10.0 (5.46-15.20)**	**38.00 (14.93-46.78) *, $p \leq 0.05$, **, $p \leq 0.05$**
TGF-β+ CD4+ / CD4+	1 mo	3.51 (1.46-4.47)	**3.54 (2.47-6.27)**	**1.30 (0.54-2.49) **, $p \leq 0.05$**

At 1 wk the prevalence of CD49d-expressing TGF-β+ CD4 lymphocytes, which are prone to enter the CNS was elevated in NAIS compared to both HIE groups, and all other time points within the NAIS group (Figure 20). By 1 mo of age however, the prevalence of TGF-β+ CD4 cells became lower in NAIS than in HIE (Table 9).

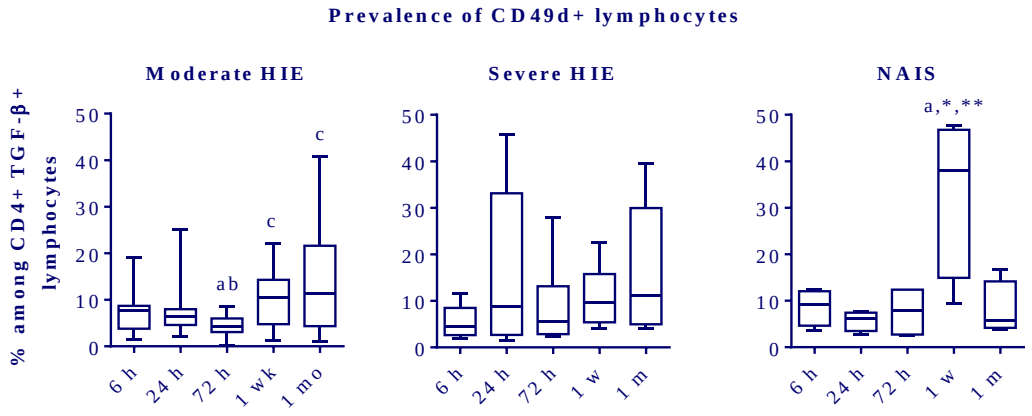

Figure 20. Alterations in the prevalence of cells expressing CD49d within the CD4+ TGF-β+ population during the first mo of life within the moderate, severe HIE and NAIS. *Moderate HIE (n = 17), severe HIE (n = 11), NAIS (n = 4). Horizontal line: median, box: interquartile range, whisker: range. $p \leq 0.05$ a vs 6 h, b vs 24 h, c vs 72 h (all within the same group); * vs moderate HIE at the same time point, ** vs severe HIE at the same time point.*

6.2.2 PLASMA CYTOKINES

Our results indicate a marked inflammatory response in NAIS at 72 hours, characterized by the elevated plasma levels of several cytokines, i.e. IL-5, IL-17 and MCP-1 compared to HIE (Table 10). Plasma MCP-1 level at 72 h was higher than at 6 h than at all other time points within the NAIS group (Figure 21). By 1 mo however, inflammatory response appears to decrease in NAIS, as plasma levels of IL-4, IL-12 and IL-17 are lower compared to HIE groups (Table 10). The level of IL-4 was lower at 1 mo than at 72 h within the NAIS group (Figure 21) (233). Plasma IL-12 and IL-17 levels showed a similar decreasing tendency at 1 mo, however this difference was not significant, probably due to the fact, that the number of cases was very low.

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Table 10. The differences in plasma cytokine levels between moderate, severe HIE and NAIS. *The plasma levels of cytokines are shown at different time points in pg/mL. Data are expressed as median and interquartile range; p≤ 0,05; *= vs moderate at the same time point, ** = vs severe at the same time point. The data, where significant differences were present compared to NAIS are shown in bold.*

	Time	Moderate HIE (n = 17)	Severe HIE (n = 11)	NAIS (n = 4)
Plasma cytokines (pg/ml)				
IL-5	72 h	1.37 (0.00-4.69)	**0.20 (0.00-0.46)**	**3.42 (1.42-6.35) **, p ≤ 0.01**
IL-17	72 h	**30.44 (26.65-51.62)**	**33.91 (29.16-48.02)**	**62.52 (51.62-645.7) *, p ≤ 0.05 **, p ≤ 0.05**
MCP-1	72 h	**251.9 (87.12-595.6)**	678.0 (214.0-2311)	**1090 (547.2-1254) *, p ≤ 0.05**
IL-4	1 mo	**1.68 (0.84-2.54)**	**1.52 (0.84-2.44)**	**0.55 (0.22-0.89) *, p ≤ 0.05 **, p ≤ 0.05**
IL-12	1mo	**18.11 (13.34-39.53)**	**21.52 (15.96-36.46)**	**11.74 (10.90-12.06) *, p ≤ 0.05 **, p ≤ 0.05**
IL-17	1mo	**32.58 (22.02-51.92)**	**31.11 (19.50-74.08)**	**14.75 (10.49-15.85) *, p ≤ 0.05 **, p ≤ 0.01**

The plasma levels of IL-1β, IL-2, IL-6, IL-7, IL8, IL-10, IL-13, IFN-γ, TNF-α, TGF-β, G-CSF, GM-CSF, MIP-1β and VCAM showed no alteration is NAIS compared with HIE.

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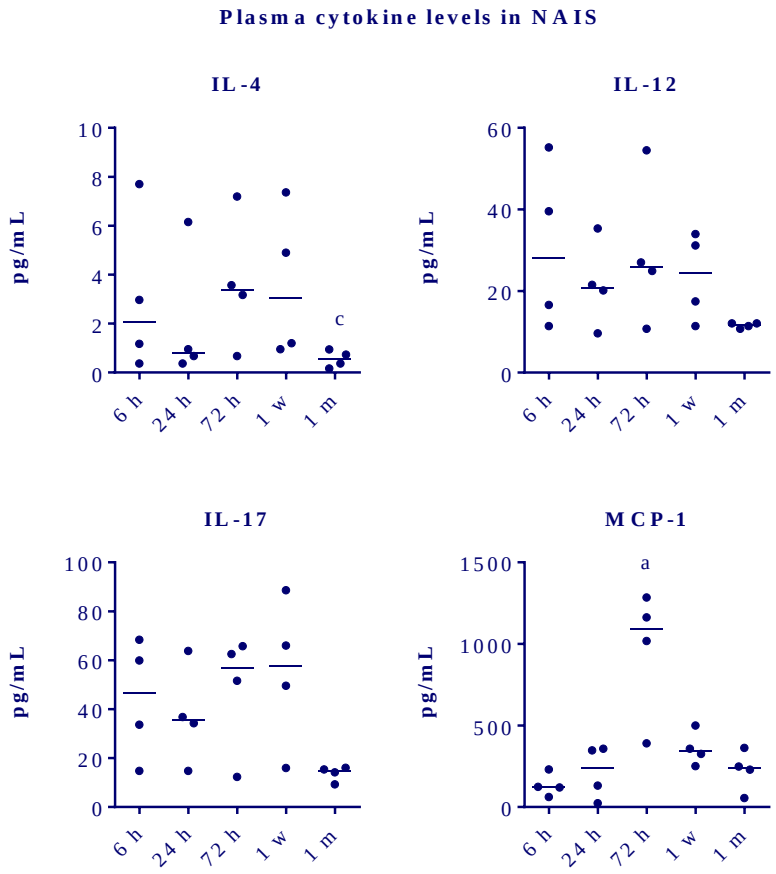

Figure 21. Alterations of the plasma cytokine levels in the first month of life in NAIS.

Plasma cytokine levels are shown in pg/mL. NAIS (n=4). Horizontal line: median. $p \leq 0.05$ a vs 6 h, b vs 24 h, c vs 72 h.

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7 DISCUSSION

7.1 COMPARISONS BETWEEN MODERATE AND SEVERE HIE

The developing brain of a neonate responds very differently to HI injury than the adult brain. Although humans are capable of withstanding far longer periods of hypoxemia during the fetal period of life, and the neonatal brain has a substantial neuroplasticity, HI injury during the perinatal period is still much more likely to lead to severe brain damage and life-long disability. This is due to the unique vulnerabilities of the immature brain hypoxia-ischemia (2). Evidence indicates, that the cytokine network plays an essential role in the normal development of the CNS (463-465). As a consequence, the developing brain is particularly susceptible to the changes of the cytokine network. In addition, the cytokine network plays a central role in the neuroinflammatory response which follow HI brain injury (393). Therefore, the alterations of the cytokine network following HI brain injury represents an area of intensive research.

The neuroinflammatory response results in complex changes in the cytokine network, which in certain cases lead to moderate HIE and good neurodevelopmental outcome, while in other cases result in severe HIE and poor outcome such as death or severe disability. We therefore aimed to understand the key features of neuroinflammation in moderate and severe HIE, especially focusing on the alterations of the adaptive immune system. Previous data mainly presented the alterations of plasma cytokine levels, which could provide a global overview of the immune response but gives no information about the characteristic changes of different immune cells. Therefore, our primary aim was to characterize the prevalence and cytokine production of T cells, however, we also measured plasma cytokine levels in order to gain a better overview.

7.1.1 PRO-INFLAMMATORY CYTOKINES

7.1.1.1 IL-1β

IL-1β is one of the most important pro-inflammatory cytokines, which appears to influence the severity of the neuroinflammatory response following HI brain injury in neonates (161). As previously detailed, there is surmounting evidence supporting the central role of IL-1β in the exacerbation of neuroinflammation, and consequently poor neurological outcome (see introduction). The detrimental consequences of elevated IL-1β production are further supported by data from human in vivo studies. In a study by Aly et al. the CSF level of IL-1β at 24 h of life was the best predictor of poor neurological outcome at 6 and 12 months after HI injury. They found a high CSF to plasma ratio of IL-1β, based on which they suggested, that local IL-1β production in the CNS is especially relevant. They concluded, that IL-1β appears to play a crucial role in the exacerbation of HI brain injury and is a marker of severe HIE (354).

In our current study, we focused on the intracellular cytokine production of T lymphocytes and also measured plasma cytokine levels to observe correlations between the two. The capacity of T cells to produce IL-1β has only recently been identified and there is still much to understand about the functional relevance of this finding (389). However, we observed significant IL-1β production in T lymphocytes after perinatal HI brain injury, which was more pronounced in severe HIE in the early phase. Both the prevalence and the extravasation of IL-1β producing CD4+ T cells was higher in severe HIE than in moderate HIE at 6 h. Based on our ROC analysis, these data could be useful in differentiating between moderate and severe HIE at an early stage (up to 6 h after birth). IL-1β-producing CD4+ T lymphocytes appear to play an important role in the initial phase of neuroinflammation, as intracellular IL-1β levels were the highest at 6 h in both groups and were comparably lower at later time points. Plasma IL-1β levels also followed similar tendencies. Our data is in line with previous studies suggesting that enhanced IL-1β production could contribute to aggravated neuronal injury. However, as intracellular concentrations of IL-1β gradually decrease in both HIE groups after 6 h, it cannot be ruled out, that a certain level of initial increase could be a physiological necessity enabling the reparative processes.

There is much data from animal models demonstrating the therapeutic benefits

of peripherally administered IL-1 receptor antagonistic agents following perinatal HI brain injury (466). IL-1 receptor antagonistic agents are in clinical use in several autoimmune disorders, and the possibility of IL-1β inhibition opens an intriguing novel area of research.

Overall, our results suggest that IL-1β may play an important role in exacerbating tissue damage in the early phase of neuroinflammation after perinatal HI brain injury. Based on the prevalence and extravasation of IL-1β producing CD4+ T cells at 6 h, the severity of HIE can be predicted early on. Although it cannot be ruled out that a certain level of initial increase in IL-1β production is necessary for the physiological reparative process, our data - in line with previous results - indicates, that excess IL-1β production correlates with more severe HI injury (284).

7.1.1.2 IL-6

Recent years' research data suggests, that IL-6 might play a dual role in the neuroinflammatory process after HI brain injury, exacerbating the inflammation in the early phase, but exerting anti-inflammatory or even neurotrophic effects in the secondary and tertiary phases (161). Previous human in vivo studies showed an association between elevated CSF levels of IL-6 after perinatal asphyxia and poor neurological outcome, cerebral palsy and death (393, 419). Martin-Ancel et al. measured CSF IL-6 levels in infants with severe HIE at 12 and 72 h and found a marked decrease during this period, which supports the detrimental effects of IL-6 in the early phase. Several authors suggest that during neuroinflammation IL-6 is primarily produced intrathecally, indicated by the high CSF to plasma ratios (393). It is important to mention however, that the majority of these results were obtained before therapeutic hypothermia was introduced, one of the most important effects of which is the rapid suppression of early cytokine response. In a previous study, Roka et al. demonstrated, that early IL-6 production (at 6 h) is suppressed by therapeutic hypothermia (246).

In the current study we only found a difference between moderate and severe HIE in the plasma level of IL-6 at 1 wk of age, where it was higher in the severe group. By 1 mo, plasma IL-6 levels decreased in moderate HIE, but not in severe HIE. In T cells, we found no differences between the two study groups in intracellular IL-6 levels

or the prevalence of IL-6+ cells. It is interesting to note however, that the intracellular level of IL-6 in CD4+ T cells peaked at 24 hours in both patient groups and declined later, supporting the role of IL-6 in the acute phase. However, the fact that we found no alterations in the IL-6 production of peripheral T cells suggests, that the local production of IL-6 could be more relevant. Another possible explanation is that hypothermia effectively suppresses the IL-6 production of T cells (284).

7.1.1.3 IL-17

IL-17 is an important pro-inflammatory cytokine primarily produced by a pro-inflammatory subset of T cells, Th17 cells. IL-17 has been shown to increase delayed neuronal damage in the chronic phase of neuroinflammation (402). In the current study, we found a continuous elevation in the prevalence of IL-17 expressing CD4+ T cells (Th17 cells) in severe HIE from 24 h until the end of the observation period. This increase was not present in moderate HIE. At 1 wk this difference between the study populations was significant, which supports the role of Th17 cells in the delayed progression of HI injury. CD4+ T lymphocytes also produced higher amounts of IL-17 in the severe group at 72 h than the moderate group. CD8+ T cells also produced higher amounts of IL-17 in severe HIE at 24 h. This delayed increase in the prevalence and cytokine production of Th17 cells could indicate their role in maintaining the chronic, latent neuroinflammation, which could influence functional regeneration unfavorably (284).

7.1.1.4 TNF-α

The amount of data supporting the deleterious effects of TNF-α during neuroinflammation is compelling (see introduction). Although TNF-α is capable of promoting cell survival in certain immunological contexts, it appears, that the receptor constitution of CNS cells during neuroinflammation is unfavorable. TNF-α has been associated with reduced oligodendrocyte survival and myelination, worse neurological outcome and higher incidence of cerebral palsy in term infants with HIE (420, 405, 421, 161).

In this study, we found a delayed increase in the TNF-α production of CD4+ T cells from 24 hours of life until the end of our observation period in both study group.

At 1 mo, the intracellular level of TNF-α in CD4+ T cells was higher in the severe group than in the moderate group, which suggests, that a sustained elevation in TNF-α production could be an important aspect of the chronic, latent neuroinflammation, contributing to the long-term consequences of HIE. In a study enrolling 7-year-old children with cerebral palsy, who were born preterm, both plasma TNF-α levels and TNF-α production of PBMCs upon stimulation was elevated compared to age-matched children, who were also born prematurely but had normal neurodevelopmental outcome. This alteration could indicate, that an enhanced pro-inflammatory response could remain even seven years after the initial phase of the neuroinflammatory response and that TNF-α could play an important role in this prolonged immune activation (118, 467).

Another interesting observation was, that at 6 hours the prevalence of TNF-α producing CD4+ T cells prone to enter the CNS (CD49d+ CD4+ TNF-α+ cells) was lower in severe HIE than at all other time points and also compared to moderate HIE. We interpreted this as a result of increased extravasation, suggesting that TNF-α producing CD4+ cells could also play a role in the early phase of the neuroinflammatory response. From 24 hours onward however, we found an increase in CD49d-expression of TNF-α+ CD4+ T cells in severe HIE, which at 72 h reached a significantly higher level than in moderate HIE. This could mean increased CD49d production in severe HIE in the chronic phase of neuroinflammation, which could lead to an enhanced capacity of CD4+ TNF-α+ cells to enter the CNS. This hypothesis, that the delayed increase in CD49d expression reflects elevated production is supported by the observations of Rothhammer et al., who showed that in mice naïve T cells (CD4+ CD44- Foxp3-) require approximately 3 days in Th1 promoting milieu to differentiate into encephalitogenic T cells, which express high levels of CD49d (468). This timeline could account for our observations and explain the initial decrease in the prevalence of CD49d+ TNF-α+ CD4+ T cells as the consequence of increased extravasation, decreasing the circulating pool of cells and the delayed elevation in the prevalence of these cells as a result of increased differentiation and CD49d production leading to an increased pool of circulating cells (284). Overall, our results suggest that TNF-α-producing CD4+ T cells play an important role during neuroinflammation, especially in the maintenance of a chronic ongoing inflammatory response.

7.1.2 ANTI-INFLAMMATORY FACTORS

We aimed to gain a comprehensive overview of the anti-inflammatory components of the adaptive immune system, and therefore measured intracellular and plasma TGF-β levels, the prevalence of Treg cells and the components of the kynurenine (KYN) pathway.

TGF-β is one of the most important immunoregulatory cytokines, inhibiting the function of pro-inflammatory cells, promoting Treg function and establishing an anti-inflammatory milieu (428-434). In addition to its immunological function, TGF-β is essential for the development, repair and survival of neurons (355). There is substantial evidence indicating that TGF-β exerts neuroprotective effects after HI brain injury, possibly by attenuating inflammation, protecting from excitotoxicity and apoptosis and promoting neuroregeneration (441). After HI injury, factors associated with reparative processes, including TGF-β have been shown to be expressed later than pro-inflammatory cytokines (355). In our study, we found elevated intracellular TGF-β levels in CD4+ T lymphocytes in moderate HIE from 24 h onwards. In the severe group, the elevation did not reach a significant level. TGF-β producing CD4+ T cells also showed a higher capacity to enter the CNS (indicated by the increased prevalence of CD49d+ cells) at 1 wk and 1 mo compared to 72 h in the moderate group, which was also not present in severe HIE. This delayed increase in TGF-β production and increased capacity for extravasation in the chronic phase of the neuroinflammation could play a role in attenuating the inflammatory response and promoting neuroregeneration in moderate HIE. The lack of this immunoregulatory effect could be an important component of the more aggravated neuroinflammatory process in severe HIE. The prevalence of Treg cells did not differ considerably between the two study groups, we only observed a moderately higher prevalence of Treg cells in severe HIE at 24 h, which could be a part of a compensatory mechanism.

The kynurenine system exerts many immunoregulatory functions and is an important link in neuro-immune interactions. It appears to be involved in the pathophysiology of a wide array of neurological disorders, however, no data has been published from perinatal HI injury previously (455). In the current study, we observed a significant increase in TRP levels in both groups by 1 mo of life. In line with this, the level of TRP catabolite KYN and further catabolite KYNA decreased in both

groups from 1 wk onwards. This indicates an enhanced IDO enzymatic activity during the first wk (calculated from the ratio of KYN to TRP) which declines by 1 mo of age in both HIE groups. However, at 1 mo of age, KYN levels were higher in moderate HIE than in severe HIE, which could be a sign of augmented anti-inflammatory processes in moderate HIE. Overall, following perinatal HI brain injury, an elevated IDO activity and TRP catabolism is present in the early postnatal period (until 1 wk of age) in both moderate and severe HIE. This could be part of a physiological immunoregulatory process, suppressing the inflammatory response to hypoxia-ischemia. However, by the end first mo after perinatal HI injury the significance of this pathway appears to decrease (284).

7.2 COMPARISONS BETWEEN HIE AND NAIS

As previously described, the inflammatory pathway appears to play an important role in the pathomechanism of NAIS. On the one hand, it appears that an inflammatory pre-conditioning could be one of the cornerstones of the development of NAIS (214), on the other hand, ischemic brain injury triggers a neuroinflammatory response, which causes the brain lesion to develop over an extended period of time. This is a common feature of HIE and NAIS and is often the time when neonates are admitted to the intensive care unit. There is evidence supporting the role of T lymphocytes in this process (232), however, hardly any data is available on the alterations of the adaptive immune responses in NAIS (See pathophysiology of NAIS). T lymphocytes are key members of the adaptive immune system, exerting their effects largely via the cytokine network. Therefore, describing the alterations of the cytokine production of T lymphocytes is an important aspect of understanding the role of the adaptive immune system in the development of NAIS. Plasma cytokine levels could also provide further insight into the immune pathology. By measuring the same parameters over the entire first month of life, it could be possible to map the dynamic changes occurring in the peripheral blood during the inflammatory process following NAIS. Although HIE and NAIS often presents with similar clinical signs and overlap, there is more and more evidence indicating, that there are also fundamental differences between these two conditions and it would be important to differentiate between them early on which is not always possible based on clinical presentation.

7.2.1 PRO-INFLAMMATORY CYTOKINES

The intracellular IL-1β production is comparable between HIE and NAIS. IL-1β also appears to play a role in the early events of the neuroinflammatory process after NAIS, indicated by the initial elevation in IL-1β production, which decreases by 24 h of age.

The intracellular IL-6 production of CD8+ lymphocytes is different in NAIS compared to HIE. At 72 h the intracellular IL-6 level of CD8+ cells is higher in severe HIE than in NAIS. However, at 1 mo whereas the IL-6 level in CD8+ cells remains elevated in NAIS patients, it decreases in both HIE groups. This finding is intriguing

in light of the emerging evidence of the dual role of IL-6 following HI injury, exerting mainly pro-inflammatory effects in the early phase and anti-inflammatory effects in the chronic phase of neuroinflammation during the regenerative processes (161). Whereas we observed no difference between the two HIE groups in IL-6 production, the elevated IL-6 production at 72 h in severe HIE compared to NAIS could reflect an aggravated inflammatory process in severe HIE. On the other hand, the sustained elevation in NAIS during the first month of life could be part of the chronic neuroregenerative process. This is in line with literary data from pediatric stroke cases, where plasma IL-6 levels were still elevated 6 months after AIS (236).

IL-17 is the characteristic cytokine produced by Th17 (CD4+), Tc17 (CD8+) cells and γδ-T cells. IL-17 producing cells have been found to be associated with acute ischemic stroke (AIS) in adults. One week after AIS the plasma level of IL-17 and the prevalence of circulating Th17 cells was elevated in adults and showed a decrease by 1 month (469). Th17 cells are also suspected to play a role in the development of HI injury. Yang et al. found a significant influx of Th17 cells into the brain tissue, if HIE occurred after LPS sensitization in rats or following a history of maternal chorioamnionitis in human neonates (403). At 6 h, there was a higher prevalence of IL-17 producing CD8+ T lymphocytes (Tc17 cells) in NAIS than in severe HIE, and CD8+ cells also produced higher amounts of IL-17 in NAIS than in moderate HIE. Tc17 cells have been shown to play a role in a variety of inflammatory diseases (401). For instance, in active brain lesions of multiple sclerosis patients, elevated IL-17 expression was present in both CD4+ and CD8+ T cells (470). We also found a higher plasma levels of IL-17 in the secondary phase of neuroinflammation (at 72h) in NAIS than in HIE. However, by 1 mo, the plasma level of IL-17 decreased in NAIS, while it remained elevated in HIE. As NAIS is suspected to develop on the base of in-utero inflammatory pre-conditioning, it is possible, that IL-17 producing T cells could play a role in the immune pathology.

In NAIS intracellular IFN-γ production of CD4+ T cells peaked at 24 h and decreased by 72 h, when it became lower than in either HIE groups. Intracellular IFN-γ levels of CD4+ T cells remained comparably lower in the later time points. IFN-γ is a characteristic Th1 cytokine, which is produced in the highest amounts by these cells. It is therefore possible, that an enhanced Th1 response is present in HIE compared to

NAIS during the secondary phase of neuroinflammation.

MCP-1 plays a central role in the activation of monocytes and is potent chemoattractant for them. MCP-1 is also suspected to play a role in the development of NAIS. In one murine model of chorioamnionitis-associated perinatal stroke, the level of MCP-1 was elevated in HI injury only if in-utero LPS sensitization was present previously (214). In our study, the level of MCP-1 was elevated in NAIS at 72 h both compared to the starting 6 h value and compared to moderate HIE, which could support the association between of in-utero inflammation and NAIS.

Overall it appears that CD8+ T cells show a slightly altered cytokine profile, with a more prominent pro-inflammatory response in NAIS. IL-17 producing cells could also be involved in the pathomechanism of NAIS, however further investigations are necessary to verify these findings and determine their relevance (233). Plasma cytokine levels also indicate a difference between NAIS and HIE. We found signs of an enhanced inflammatory response at 72 h in NAIS compared to HIE, when the level of IL-17 and MCP-1 were elevated in NAIS.

7.2.2 ANTI-INFLAMMATORY CYTOKINES

The anti-inflammatory pathways also show some specific characteristics in NAIS. IL-10 is a characteristic cytokine produced by regulatory T cells, which exerts a wide array of anti-inflammatory effects and has been shown to be neuroprotective after HI injury (426, 406). Trandem et al. were however recently also able to demonstrate in a murine model of virus encephalitis, that CD8+ T lymphocytes (CTLs) are also capable of producing significant amounts of IL-10 at the peak of their activation. They concluded, that CTL-derived IL-10 is most likely to play an important autoregulatory role when the activation of these cells reaches a certain level, preventing local tissue destruction. The expression IL-10 decreased rapidly after 1 wk in CTLs in this experiment, whereas CD4+ T cells expressed IL-10 for a much longer time, up to at least 42 days (471). In our current study we found a higher prevalence of IL-10 expressing CD8+ lymphocytes in severe HIE than in NAIS during the first 72 hours of life. The prevalence of IL-10 expressing CTLs was also slightly higher in moderate HIE than in NAIS, but this did not reach a significant level. On the other hand, the IL-10 production of CD4+ lymphocytes was higher in NAIS than in

moderate HIE at 24 h.

One explanation for this finding could be, that in severe HIE, the more global and aggravated inflammatory response evokes a higher level of CTL-activation as well, which in return produce more IL-10. Another, more plausible explanation could be, that there is a difference in the onset of the two disorders. Whereas evidence supports, that the majority of events causing global asphyxia occur during or around the birth, NAIS is likely to be preceded by a longer period of ongoing inflammation. In this case, the explanation could be, that the IL-10 production of CD8+ T cells decreases in NAIS by the time of birth. This would also give a reasonable explanation to the elevated prevalence of IL-10 producing CD4+ T cells, since Th cells are known to produce IL-10 for a much longer time. Altogether the IL-10 production of T cell subsets also appears to be different in NAIS and HIE and could also suggest an earlier onset of inflammation in NAIS.

Another intriguing finding is that the prevalence of TGF-β-producing Th cells prone to enter the CNS (CD49d-expressing TGF-β+ CD4+ T cells) was significantly higher at 1 wk in NAIS compared to 6 h, and also compared to both HIE groups. The prevalence of these cells shows an increase within the moderate HIE group after 72 h, but still reaches a significantly higher level in NAIS. TGF-β is the primary cytokine of Treg cells and is one of the most important neuroprotective cytokines, which also plays a role in neuroregeneration. Thus, it appears, that there is a marked peak in immunoregulatory cells entering the CNS 1 wk in NAIS. By 1 mo however, the prevalence of circulating TGF-β-producing Th cells decreases in NAIS and becomes lower compared to both HIE groups. This is in line with the alterations of the plasma cytokine levels, which also indicate a general attenuation of the inflammatory response by 1 mo in NAIS compared to HIE.

Among the plasma cytokines, the level of IL-5 was in NAIS than in HIE. IL-5 is a Th2 associated cytokine, the elevation of which could be part of a generally enhanced inflammatory response, which could also be a sign of the beginning of the reparative phase. By 1 mo we saw a general decrease in the plasma level of several cytokines, including Th2 associated cytokines such as IL-4 in NAIS compared to HIE. This indicates, that there is a general moderation of the inflammatory process in NAIS compared to HIE (233).

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7.3 STUDY LIMITATIONS

The main limitation of our study is the lack of a healthy control group. Although this would have provided further insight into the physiological adaptation of the immune system during the first month of life and into the alterations which are cause by HIE, due to the sophisticated study design and the high number of samplings, this could not have been carried out ethically in healthy neonates. Although the above-mentioned data would be scientifically interesting, the real clinical relevance of such data is questionable. On the one hand, there are several studies providing such data from individual time points, on the other hand, the real clinical question is the factors which differentiate between a moderate and a severe insult. Therefore, we believe that our study is still able to provide relevant data, even if the comparisons are made between two patient populations. Another limitation is the lack of data from the local milieu from CSF or brain samples, however, the current clinical practice no longer warrants CSF sampling in HIE. A further limitation of the study is that five neonates in each study group had to be administered hydrocortisone as part of blood pressure support, the impact of which on their cytokine production cannot be ruled out. This question needs further investigation.

Clearly, the main limitation regarding the comparisons made between HIE and NAIS is the very small number of cases. Therefore, these data must be considered as incentive for future, larger case-control studies. We observed a notable variability in certain plasma cytokine levels within this small group of patients, which could be due to the relative instability of plasma cytokines. It is possible, that with increasing the number of cases, this will decrease, however, plasma cytokine levels tend to show greater variability than intracellular levels. We see the value of these results in providing a base for future clinical research on differentiating factors between the two distinct etiologies (global hypoxic-ischemic encephalopathy and perinatal arterial ischemic stroke), which often overlap clinically. Since gaining a better understanding of the pathophysiology of NAIS is a primary focus in this field, and no such serial data have been previously published in humans, despite the small number of cases, these data could provide a novel insight into the immunological processes following NAIS (233).

8 CONCLUSIONS

In conclusion, the involvement of the cytokine network in the development of perinatal HI injury is supported by a rapidly expanding body of evidence. Understanding the alterations of this complex network of neuro-immune interactions are a key focus in current research, with special attention on prognostic markers and options for individualized therapy. Another important focus is understanding the tertiary, latent phase of neuroinflammation and its contribution to the development of HI injury, as this period could be especially important for intervention.

With regards to the alterations of T lymphocytes in perinatal HIE, IL-1β and IL-6 producing T lymphocytes appear to play a key role in the adaptive immune response during the early phase of neuroinflammation, whereas prolonged TNF-α-production of T cells could be an important component of the latent, chronic phase of neuroinflammation. The prevalence of Th17 cells was elevated in severe HIE from 24 h onwards (compared to 6 h) and at 1 wk it was significantly higher than in moderate HIE, which suggests, that Th17 cells could also play a role in delayed progression of injury. Elevating TGF-β levels could be important in regulating the inflammatory response and initiating neuroregeneration (Table 11). By assessing the prevalence of peripheral IL-1β expressing CD4+ cells and the CD49d expression of these cells at 6 h, it was possible to differentiate between moderate and severe HIE at an early stage (284).

To conclude our findings in NAIS, our data indicate an enhanced inflammatory response at 72 h in NAIS compared to HIE, characterized by the elevation several cytokines (i. e. IL-5, IL-17, MCP-1). At 1 week there is a marked increase in the extravasation of TGF-β producing CD4+ T cells in NAIS both compared to the 6 h value and HIE, which could indicate an earlier initiation of the reparative process. It appears that the inflammatory response becomes attenuated by 1 month, which is indicated by the decrease in plasma cytokine levels (i. e. IL-4, IL-12, IL-17) and the prevalence of TGF-β producing CD4+ T cells. Differences in the levels of cytokines which have been shown to link systemic inflammatory response with neuroinflammation, such as IL-17 and MCP-1 were present in NAIS. These data appear to support the hypothesis of an ongoing in-utero inflammation prior to NAIS, but larger case-control studies are pivotal to interpret the value of these findings.

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8.1 KEY FINDINGS

1) IL-1β and IL-6 producing T lymphocytes appear to play an important role in the early phase of the adaptive immune response in perinatal HIE.
2) TNF-α production is sustained during the first month of life in T cells and is higher in severe HIE, which could contribute to a worse outcome
3) The elevated prevalence of Th17 lymphocytes in severe HIE could indicate the role of this subset in the delayed progression of HI brain injury
4) Elevating TGF-β production and increased extravasation CD4+ T could play an important regulatory role in HIE by initiating the reparative processes
5) The assessment of the prevalence and CD49d expression of IL-1β+ CD4+ cells at 6 h appears to be able to predict severity at an early stage in HIE
6) In NAIS the inflammatory response is more enhanced at 72 h than in HIE, indicated by the higher level of several plasma cytokines (i. e. IL-5, IL-17, MCP-1).
7) At 1 wk, there is a marked increase in the extravasation of TGF-β producing CD4+ T cells in NAIS both compared to the 6 h value and HIE, which could indicate an enhanced reparative process
8) By 1 mo the inflammatory response is attenuated in NAIS, indicated by lower plasma cytokine levels and the lower prevalence of TGF-β producing CD4+ T cells

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Table 11. Summary of the proposed effect of cytokines on the course of hypoxic ischemic encephalopathy following perinatal asphyxia

Pro-inflammatory		**Anti-inflammatory**	
Contribution to better outcome			
G-CSF	rapid decrease in moderate insult, higher plasma levels in severe insult	**TGF-β**	increased production and extravasation in moderate insult
		IDO	early compensatory role, up to 1 wk
Contribution to worse outcome			
IL-1β	higher initial prevalence and extravasation in severe insult	**Treg**	Unremarkable difference at 24 h, lack of regulatory effect
IL-6	highest level at 24 h, higher plasma levels in severe HIE at 1 wk, decrease in moderate HIE by 1 mo		
IL-17	elevated prevalence in severe HIE up to 1 mo		
TNF-α	elevated intracellular levels up to 1 mo, higher in severe HIE		

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9 SUMMARY

Perinatal asphyxia and following hypoxic-ischemic encephalopathy (HIE) is one of the leading causes of child mortality and long-term disability in the world. The perinatal period also carries the highest risk for acute ischemic stroke (NAIS) in the entire childhood, which is one of the leading causes of cerebral palsy. Neuroinflammation is one of the most important features of perinatal HI brain injury. The severity of the neuroinflammatory response plays a key role in determining the degree of damage and ultimately the outcome. Neuroinflammation may have dual aspects being a hindrance, but also necessary for the recovery of the CNS.

In this study, our aims were to characterize the alterations of the intracellular cytokine production of T-lymphocytes and plasma cytokine levels during the first month of life in HIE and NAIS:

We analysed data from 32 term neonates requiring moderate systemic hypothermia in a single-centre observational study. Blood samples were collected on five occasions, between 3-6 h of life, at 24 h, 72 h, 1 wk and 1 mo of life. Neonates were divided into a moderate (n = 17) and a severe (n = 11) group based on neuroradiological and aEEG characteristics. Four neonates were diagnosed with NAIS by MRI. Peripheral blood mononuclear cells were assessed with flow cytometry. Cytokine plasma levels were measured using Bioplex immunoassays. Components of the kynurenine pathway were assessed by HPLC.

In HIE IL-1β and IL-6 appear to play a key role in the adaptive immune response during the early phase of neuroinflammation, whereas elevated prevalence of Th17 cells and prolonged TNF-α-production of T cells could be important aspects of the latent, chronic phase of neuroinflammation. Based on ROC analysis, the assessment of the prevalence and extravasation of IL-1β-producing CD4+ lymphocytes could predict the severity of HIE at 6 h. Increasing TGF-β production of CD4+ T cells could indicate their role in regulating the inflammation and initiating the reparative processes. In NAIS the plasma levels of several cytokines were elevated at 72 h. At 1 wk, the extravasation of TGF-β producing CD4+ T cells was higher in NAIS which suggests the initiation of the reparative process. The inflammatory response appears to become moderated by 1 mo in NAIS. In NAIS we observed changes in several cytokines which could support the involvement of an in-utero inflammatory response.

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10 ÖSSZEFOGLALÁS

A perinatális asphyxia és a következményes hypoxiás-ischaemiás encephalopathia (HIE) a gyermekkori mortalitás és a maradandó idegrendszeri károsodás vezető oka. A perinatális időszak kiemelt kockázatot hordoz neonatális artériás ischaemiás stroke (NAIS) szempontjából is, amely a cerebrális parézis egyik legfontosabb oka. A neuroinflammáció alapvető meghatározója mindkét kórképhez kötötten bekövetkező károsodásnak. A neuroinflammatorikus válasz lefolyása meghatározza a központi idegrendszer károsodásának a súlyosságát.

A célkitűzésünk a T limfociták prevalenciájának és citokin-termelésének, valamint a citokinek plazmaszintjének a jellemzése volt HIE-t és NAIS-t követően.

A vizsgálatunkba 32 érett újszülöttet vontunk be, akik megfeleltek a mérsékelt, teljes test-hipotermia terápiás kritériumrendszerének. Összesen 5 alkalommal vettünk perifériás vérmintát az újszülöttektől 6, 24 és 72 órával a születést követően, valamint egy hetes, illetve 1 hónapos korban. A mérsékelt (n = 17) és súlyos HIE (n = 11) csoportok az aEEG és MRI vizsgálatok eredményei alapján utólag kerültek kialakításra. Négy újszülött esetében az MRI vizsgálat elvégzését követően NAIS igazolódott. A perifériás mononukleáris sejteket áramlási citometriával vizsgáltuk. A citokinek plazma szintjének meghatározására Bioplex immunassay-t alkalmaztunk. A kinurenin útvonal komponenseinek a szintjét HPLC segítségével mértük.

A vizsgálataink azt támasztják alá, hogy a HIE-hez kapcsolódó neuroinflammáció során az IL-1β és az IL-6 alapvető szerepet játszik az adaptív immunválaszban a korai fázisban. ROC analízis alapján az IL-1β-t termelő CD4+ limfociták prevalenciájának és extravazációjának vizsgálata révén már korai fázisban (6 óránál) elkülöníthető volt a két csoport. Ezzel szemben a Th17 sejtek emelkedett prevalenciája, valamint a CD4+ T sejtek tartósan emelkedett TNF-α termelése fontos elemei lehetnek a neuroinflammáció krónikus fázisának. A fokozatosan emelkedő TGF-β termelés révén a CD4+ T sejtek fontos regulátoros szerepet játszhatnak és hozzájárulhatnak a KIR regeneráció megindulásához. NAIS-ban 72 órás korban számos citokin plazmaszintje emelkedett volt. Egy hetes korban a TGF-β termelő CD4+ sejtek extravazációja magasabb volt NAIS-ban, amely a regenerációs folyamatok korábbi megindulását jelezheti. NAIS esetében több olyan citokin szintjében észleltünk változást, amelyek felvetik az in-utero gyulladás lehetséges szerepét.

11 REFERENCES

1. Alvarez-Diaz A, Hilario E, de Cerio FG, Valls-i-Soler A, Alvarez-Diaz FJ. (2007) Hypoxic-ischemic injury in the immature brain--key vascular and cellular players. Neonatology, 92: 227-235.
2. Rocha-Ferreira E, Hristova M. (2016) Plasticity in the Neonatal Brain following Hypoxic-Ischaemic Injury. Neural Plast, 2016: 4901014.
3. Johnston MV, Trescher WH, Taylor GA. (1995) Hypoxic and ischemic central nervous system disorders in infants and children. Adv Pediatr, 42: 1-45.
4. Johnston MV, Trescher WH, Ishida A, Nakajima W. (2001) Neurobiology of hypoxic-ischemic injury in the developing brain. Pediatr Res, 49: 735-741.
5. Albensi BC, Schweizer MP, Rarick TM, Filloux F. (1999) Unilateral hypoxic-ischemic injury in the neonatal rat brain evaluated by in vivo MRI. Correlation with histopathology and neuroprotection by MK-801. Invest Radiol, 34: 249-261.
6. Sarnat HB, Sarnat MS. (1976) Neonatal encephalopathy following fetal distress. A clinical and electroencephalographic study. Arch Neurol, 33: 696-705.
7. Towfighi J, Zec N, Yager J, Housman C, Vannucci RC. (1995) Temporal evolution of neuropathologic changes in an immature rat model of cerebral hypoxia: a light microscopic study. Acta Neuropathol, 90: 375-386.
8. Baburamani AA, Ek CJ, Walker DW, Castillo-Melendez M. (2012) Vulnerability of the developing brain to hypoxic-ischemic damage: contribution of the cerebral vasculature to injury and repair? Front Physiol, 3: 424.
9. Barkovich AJ. (2005) Magnetic resonance techniques in the assessment of myelin and myelination. J Inherit Metab Dis, 28: 311-343.
10. Keunen H, Blanco CE, van Reempts JL, Hasaart TH. (1997) Absence of neuronal damage after umbilical cord occlusion of 10, 15, and 20 minutes in midgestation fetal sheep. Am J Obstet Gynecol, 176: 515-520.
11. Mallard EC, Williams CE, Johnston BM, Gluckman PD. (1994) Increased vulnerability to neuronal damage after umbilical cord occlusion in fetal sheep with advancing gestation. Am J Obstet Gynecol, 170: 206-214.
12. Bennet L, Rossenrode S, Gunning MI, Gluckman PD, Gunn AJ. (1999) The cardiovascular and cerebrovascular responses of the immature fetal sheep to acute umbilical cord occlusion. J Physiol, 517 (Pt 1): 247-257.
13. Van den Broeck C, Himpens E, Vanhaesebrouck P, Calders P, Oostra A. (2008) Influence of gestational age on the type of brain injury and neuromotor outcome in high-risk neonates. Eur J Pediatr, 167: 1005-1009.
14. Wigglesworth JS, Pape KE. (1978) An integrated model for haemorrhagic and ischaemic lesions in the newborn brain. Early Hum Dev, 2: 179-199.
15. Rivkin MJ. (1997) Hypoxic-ischemic brain injury in the term newborn. Neuropathology, clinical aspects, and neuroimaging. Clin Perinatol, 24: 607-625.
16. Volpe JJ. (1997) Brain injury in the premature infant. Neuropathology, clinical aspects, pathogenesis, and prevention. Clin Perinatol, 24: 567-587.
17. Takashima S, Tanaka K. (1978) Development of cerebrovascular architecture and its relationship to periventricular leukomalacia. Arch Neurol, 35: 11-16.

18. Takashima S, Tanaka K. (1978) Microangiography and vascular permeability of the subependymal matrix in the premature infant. Can J Neurol Sci, 5: 45-50.
19. Counsell SJ, Rutherford MA, Cowan FM, Edwards AD. (2003) Magnetic resonance imaging of preterm brain injury. Arch Dis Child Fetal Neonatal Ed, 88: F269-274.
20. Back SA, Luo NL, Borenstein NS, Levine JM, Volpe JJ, Kinney HC. (2001) Late oligodendrocyte progenitors coincide with the developmental window of vulnerability for human perinatal white matter injury. J Neurosci, 21: 1302-1312.
21. Barkovich AJ, Westmark K, Partridge C, Sola A, Ferriero DM. (1995) Perinatal asphyxia: MR findings in the first 10 days. AJNR Am J Neuroradiol, 16: 427-438.
22. Hoon AH, Jr., Reinhardt EM, Kelley RI, Breiter SN, Morton DH, Naidu SB, Johnston MV. (1997) Brain magnetic resonance imaging in suspected extrapyramidal cerebral palsy: observations in distinguishing genetic-metabolic from acquired causes. J Pediatr, 131: 240-245.
23. Maller AI, Hankins LL, Yeakley JW, Butler IJ. (1998) Rolandic type cerebral palsy in children as a pattern of hypoxic-ischemic injury in the full-term neonate. Journal of Child Neurology, 13: 313-321.
24. Menkes JH, Curran J. (1994) Clinical and MR correlates in children with extrapyramidal cerebral palsy. AJNR Am J Neuroradiol, 15: 451-457.
25. Pasternak JF, Gorey MT. (1998) The syndrome of acute near-total intrauterine asphyxia in the term infant. Pediatr Neurol, 18: 391-398.
26. Roland EH, Poskitt K, Rodriguez E, Lupton BA, Hill A. (1998) Perinatal hypoxic-ischemic thalamic injury: clinical features and neuroimaging. Ann Neurol, 44: 161-166.
27. Rutherford MA, Pennock JM, Counsell SJ, Mercuri E, Cowan FM, Dubowitz LM, Edwards AD. (1998) Abnormal magnetic resonance signal in the internal capsule predicts poor neurodevelopmental outcome in infants with hypoxic-ischemic encephalopathy. Pediatrics, 102: 323-328.
28. Johnston MV, Hoon AH, Jr. (2000) Possible mechanisms in infants for selective basal ganglia damage from asphyxia, kernicterus, or mitochondrial encephalopathies. J Child Neurol, 15: 588-591.
29. Alexander GE, Crutcher MD. (1990) Functional architecture of basal ganglia circuits: neural substrates of parallel processing. Trends Neurosci, 13: 266-271.
30. Rutherford M, Malamateniou C, McGuinness A, Allsop J, Biarge MM, Counsell S. (2010) Magnetic resonance imaging in hypoxic-ischaemic encephalopathy. Early Hum Dev, 86: 351-360.
31. McDonald JW, Johnston MV. (1990) Physiological and pathophysiological roles of excitatory amino acids during central nervous system development. Brain Res Brain Res Rev, 15: 41-70.
32. Olney JW, Collins RC, Sloviter RS. (1986) Excitotoxic mechanisms of epileptic brain damage. Adv Neurol, 44: 857-877.
33. Williams CE, Gunn AJ, Mallard C, Gluckman PD. (1992) Outcome after ischemia in the developing sheep brain: an electroencephalographic and histological study. Ann Neurol, 31: 14-21.
34. Magistretti PJ, Pellerin L, Rothman DL, Shulman RG. (1999) Energy on

demand. Science, 283: 496-497.
35. Blennow M, Ingvar M, Lagercrantz H, Stone-Elander S, Eriksson L, Forssberg H, Ericson K, Flodmark O. (1995) Early [18F]FDG positron emission tomography in infants with hypoxic-ischaemic encephalopathy shows hypermetabolism during the postasphyctic period. Acta Paediatr, 84: 1289-1295.
36. Morita-Fujimura Y, Fujimura M, Yoshimoto T, Chan PH. (2001) Superoxide during reperfusion contributes to caspase-8 expression and apoptosis after transient focal stroke. Stroke, 32: 2356-2361.
37. O'Brien JS, Sampson EL. (1965) Lipid composition of the normal human brain: gray matter, white matter, and myelin. J Lipid Res, 6: 537-544.
38. Halliwell B. (1992) Reactive oxygen species and the central nervous system. J Neurochem, 59: 1609-1623.
39. Ikonomidou C, Kaindl AM. (2011) Neuronal death and oxidative stress in the developing brain. Antioxid Redox Signal, 14: 1535-1550.
40. McLean C, Ferriero D. (2004) Mechanisms of hypoxic-ischemic injury in the term infant. Semin Perinatol, 28: 425-432.
41. Miller SL, Wallace EM, Walker DW. (2012) Antioxidant therapies: a potential role in perinatal medicine. Neuroendocrinology, 96: 13-23.
42. Mishra OP, Delivoria-Papadopoulos M. (1999) Cellular mechanisms of hypoxic injury in the developing brain. Brain Res Bull, 48: 233-238.
43. Vannucci SJ, Hagberg H. (2004) Hypoxia-ischemia in the immature brain. J Exp Biol, 207: 3149-3154.
44. Beltran B, Mathur A, Duchen MR, Erusalimsky JD, Moncada S. (2000) The effect of nitric oxide on cell respiration: A key to understanding its role in cell survival or death. Proc Natl Acad Sci U S A, 97: 14602-14607.
45. Tan S, Zhou F, Nielsen VG, Wang Z, Gladson CL, Parks DA. (1998) Sustained hypoxia-ischemia results in reactive nitrogen and oxygen species production and injury in the premature fetal rabbit brain. J Neuropathol Exp Neurol, 57: 544-553.
46. Blomgren K, Hagberg H. (2006) Free radicals, mitochondria, and hypoxia-ischemia in the developing brain. Free Radic Biol Med, 40: 388-397.
47. Castillo-Melendez M, Chow JA, Walker DW. (2004) Lipid peroxidation, caspase-3 immunoreactivity, and pyknosis in late-gestation fetal sheep brain after umbilical cord occlusion. Pediatr Res, 55: 864-871.
48. Robertson CL, Scafidi S, McKenna MC, Fiskum G. (2009) Mitochondrial mechanisms of cell death and neuroprotection in pediatric ischemic and traumatic brain injury. Exp Neurol, 218: 371-380.
49. Forstermann U, Sessa WC. (2012) Nitric oxide synthases: regulation and function. Eur Heart J, 33: 829-837, 837a-837d.
50. Ferriero DM. (2004) Neonatal brain injury. N Engl J Med, 351: 1985-1995.
51. Ferriero DM, Arcavi LJ, Sagar SM, McIntosh TK, Simon RP. (1988) Selective sparing of NADPH-diaphorase neurons in neonatal hypoxia-ischemia. Ann Neurol, 24: 670-676.
52. Ferriero DM, Arcavi LJ, Simon RP. (1990) Ontogeny of excitotoxic injury to nicotinamide adenine dinucleotide phosphate diaphorase reactive neurons in the neonatal rat striatum. Neuroscience, 36: 417-424.
53. Black SM, Bedolli MA, Martinez S, Bristow JD, Ferriero DM, Soifer SJ.

(1995) Expression of neuronal nitric oxide synthase corresponds to regions of selective vulnerability to hypoxia-ischaemia in the developing rat brain. Neurobiol Dis, 2: 145-155.

54. Ferriero D, Ashwal S. Effects of nitric oxide on neuronal and cerebrovascular function. In: SS Donn SM, Chiswick ML (szerk.), Birth Asphyxia and the Brain: Basic Science Clinical Implications. Armonk, NY: Futura Publishing, 2002: 153-188.
55. Aarts MM, Tymianski M. (2003) Novel treatment of excitotoxicity: targeted disruption of intracellular signalling from glutamate receptors. Biochem Pharmacol, 66: 877-886.
56. Ferriero DM, Holtzman DM, Black SM, Sheldon RA. (1996) Neonatal mice lacking neuronal nitric oxide synthase are less vulnerable to hypoxic-ischemic injury. Neurobiol Dis, 3: 64-71.
57. Hamada Y, Hayakawa T, Hattori H, Mikawa H. (1994) Inhibitor of nitric oxide synthesis reduces hypoxic-ischemic brain damage in the neonatal rat. Pediatr Res, 35: 10-14.
58. van den Tweel ER, Peeters-Scholte CM, van Bel F, Heijnen CJ, Groenendaal F. (2002) Inhibition of nNOS and iNOS following hypoxia-ischaemia improves long-term outcome but does not influence the inflammatory response in the neonatal rat brain. Dev Neurosci, 24: 389-395.
59. Blaschke AJ, Staley K, Chun J. (1996) Widespread programmed cell death in proliferative and postmitotic regions of the fetal cerebral cortex. Development, 122: 1165-1174.
60. Li Y, Powers C, Jiang N, Chopp M. (1998) Intact, injured, necrotic and apoptotic cells after focal cerebral ischemia in the rat. J Neurol Sci, 156: 119-132.
61. McDonald JW, Behrens MI, Chung C, Bhattacharyya T, Choi DW. (1997) Susceptibility to apoptosis is enhanced in immature cortical neurons. Brain Res, 759: 228-232.
62. Hu BR, Liu CL, Ouyang Y, Blomgren K, Siesjo BK. (2000) Involvement of caspase-3 in cell death after hypoxia-ischemia declines during brain maturation. J Cereb Blood Flow Metab, 20: 1294-1300.
63. Nakajima W, Ishida A, Lange MS, Gabrielson KL, Wilson MA, Martin LJ, Blue ME, Johnston MV. (2000) Apoptosis has a prolonged role in the neurodegeneration after hypoxic ischemia in the newborn rat. J Neurosci, 20: 7994-8004.
64. Graham EM, Sheldon RA, Flock DL, Ferriero DM, Martin LJ, O'Riordan DP, Northington FJ. (2004) Neonatal mice lacking functional Fas death receptors are resistant to hypoxic-ischemic brain injury. Neurobiol Dis, 17: 89-98.
65. van Landeghem FK, Felderhoff-Mueser U, Moysich A, Stadelmann C, Obladen M, Bruck W, Buhrer C. (2002) Fas (CD95/Apo-1)/Fas ligand expression in neonates with pontosubicular neuron necrosis. Pediatr Res, 51: 129-135.
66. McKinstry RC, Miller JH, Snyder AZ, Mathur A, Schefft GL, Almli CR, Shimony JS, Shiran SI, Neil JJ. (2002) A prospective, longitudinal diffusion tensor imaging study of brain injury in newborns. Neurology, 59: 824-833.
67. Blumberg RM, Cady EB, Wigglesworth JS, McKenzie JE, Edwards AD. (1997) Relation between delayed impairment of cerebral energy metabolism

and infarction following transient focal hypoxia-ischaemia in the developing brain. Exp Brain Res, 113: 130-137.

68. Lorek A, Takei Y, Cady EB, Wyatt JS, Penrice J, Edwards AD, Peebles D, Wylezinska M, Owen-Reece H, Kirkbride V, et al. (1994) Delayed ("secondary") cerebral energy failure after acute hypoxia-ischemia in the newborn piglet: continuous 48-hour studies by phosphorus magnetic resonance spectroscopy. Pediatr Res, 36: 699-706.
69. Williams CE, Gunn A, Gluckman PD. (1991) Time course of intracellular edema and epileptiform activity following prenatal cerebral ischemia in sheep. Stroke, 22: 516-521.
70. Ma J, Endres M, Moskowitz MA. (1998) Synergistic effects of caspase inhibitors and MK-801 in brain injury after transient focal cerebral ischaemia in mice. Br J Pharmacol, 124: 756-762.
71. Wang KK, Yuen PW. (1994) Calpain inhibition: an overview of its therapeutic potential. Trends Pharmacol Sci, 15: 412-419.
72. Johnston MV, Ishida A, Ishida WN, Matsushita HB, Nishimura A, Tsuji M. (2009) Plasticity and injury in the developing brain. Brain Dev, 31: 1-10.
73. Jung WB, Im GH, Chung JJ, Ahn SY, Jeon TY, Chang YS, Park WS, Kim JH, Kim KS, Lee JH. (2016) Neuroplasticity for spontaneous functional recovery after neonatal hypoxic ischemic brain injury in rats observed by functional MRI and diffusion tensor imaging. Neuroimage, 126: 140-150.
74. Basu AP. (2014) Early intervention after perinatal stroke: opportunities and challenges. Dev Med Child Neurol, 56: 516-521.
75. Friel K, Chakrabarty S, Kuo HC, Martin J. (2012) Using motor behavior during an early critical period to restore skilled limb movement after damage to the corticospinal system during development. J Neurosci, 32: 9265-9276.
76. Salimi I, Friel KM, Martin JH. (2008) Pyramidal tract stimulation restores normal corticospinal tract connections and visuomotor skill after early postnatal motor cortex activity blockade. J Neurosci, 28: 7426-7434.
77. Donega V, van Velthoven CT, Nijboer CH, Kavelaars A, Heijnen CJ. (2013) The endogenous regenerative capacity of the damaged newborn brain: boosting neurogenesis with mesenchymal stem cell treatment. J Cereb Blood Flow Metab, 33: 625-634.
78. Kadam SD, Mulholland JD, McDonald JW, Comi AM. (2008) Neurogenesis and neuronal commitment following ischemia in a new mouse model for neonatal stroke. Brain Res, 1208: 35-45.
79. Derugin N, Dingman A, Wendland MF, Fox C, Bollen A, Vexler ZS. (2005) Magnetic resonance imaging as a surrogate measure for histological sub-chronic endpoint in a neonatal rat stroke model. Brain Res, 1066: 49-56.
80. Johnston MV. (2009) Plasticity in the developing brain: implications for rehabilitation. Dev Disabil Res Rev, 15: 94-101.
81. Chen H, Epstein J, Stern E. (2010) Neural plasticity after acquired brain injury: evidence from functional neuroimaging. PM R, 2: S306-312.
82. Murphy TH, Corbett D. (2009) Plasticity during stroke recovery: from synapse to behaviour. Nat Rev Neurosci, 10: 861-872.
83. Giza CC, Prins ML. (2006) Is being plastic fantastic? Mechanisms of altered plasticity after developmental traumatic brain injury. Dev Neurosci, 28: 364-379.

84. Johnston MV, Nakajima W, Hagberg H. (2002) Mechanisms of hypoxic neurodegeneration in the developing brain. Neuroscientist, 8: 212-220.
85. Kurinczuk JJ, White-Koning M, Badawi N. (2010) Epidemiology of neonatal encephalopathy and hypoxic-ischaemic encephalopathy. Early Hum Dev, 86: 329-338.
86. Nelson KB, Leviton A. (1991) How much of neonatal encephalopathy is due to birth asphyxia? Am J Dis Child, 145: 1325-1331.
87. Volpe JJ. (2001) Neurology of the newborn, 4th ed: 217-276.
88. Azzopardi D, Brocklehurst P, Edwards D, Halliday H, Levene M, Thoresen M, Whitelaw A, Group TS. (2008) The TOBY Study. Whole body hypothermia for the treatment of perinatal asphyxial encephalopathy: a randomised controlled trial. BMC Pediatr, 8: 17.
89. Lawn JE, Cousens S, Zupan J, Lancet Neonatal Survival Steering T. (2005) 4 million neonatal deaths: when? Where? Why? Lancet, 365: 891-900.
90. Casey BM, McIntire DD, Leveno KJ. (2001) The continuing value of the Apgar score for the assessment of newborn infants. N Engl J Med, 344: 467-471.
91. Groenendaal F, van Bel F. Clinical Aspects and Treatment of the Hypoxic-Ischemic Syndrome. In: G Buonocore, R Bracci ,M Weindling (szerk.), Neonatology. Springer International Publishing, Cham, 2016: 1-20.
92. Beckstrom AC, Humston EM, Snyder LR, Synovec RE, Juul SE. (2011) Application of comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry method to identify potential biomarkers of perinatal asphyxia in a non-human primate model. J Chromatogr A, 1218: 1899-1906.
93. Stanley FJ, Watson L. (1992) Trends in perinatal mortality and cerebral palsy in Western Australia, 1967 to 1985. BMJ, 304: 1658-1663.
94. Locatelli A, Incerti M, Paterlini G, Doria V, Consonni S, Provero C, Ghidini A. (2010) Antepartum and intrapartum risk factors for neonatal encephalopathy at term. Am J Perinatol, 27: 649-654.
95. Cowan F, Rutherford M, Groenendaal F, Eken P, Mercuri E, Bydder GM, Meiners LC, Dubowitz LM, de Vries LS. (2003) Origin and timing of brain lesions in term infants with neonatal encephalopathy. Lancet, 361: 736-742.
96. Badawi N, Kurinczuk JJ, Keogh JM, Alessandri LM, O'Sullivan F, Burton PR, Pemberton PJ, Stanley FJ. (1998) Intrapartum risk factors for newborn encephalopathy: the Western Australian case-control study. BMJ, 317: 1554-1558.
97. Ellis M, Manandhar N, Manandhar DS, Costello AM. (2000) Risk factors for neonatal encephalopathy in Kathmandu, Nepal, a developing country: unmatched case-control study. BMJ, 320: 1229-1236.
98. Badawi N, Kurinczuk JJ, Keogh JM, Alessandri LM, O'Sullivan F, Burton PR, Pemberton PJ, Stanley FJ. (1998) Antepartum risk factors for newborn encephalopathy: the Western Australian case-control study. BMJ, 317: 1549-1553.
99. Gunn AJ, Parer JT, Mallard EC, Williams CE, Gluckman PD. (1992) Cerebral histologic and electrocorticographic changes after asphyxia in fetal sheep. Pediatr Res, 31: 486-491.
100. Jensen A, Garnier Y, Berger R. (1999) Dynamics of fetal circulatory responses

to hypoxia and asphyxia. Eur J Obstet Gynecol Reprod Biol, 84: 155-172.
101. Vannucci RC. (1990) Experimental biology of cerebral hypoxia-ischemia: relation to perinatal brain damage. Pediatr Res, 27: 317-326.
102. Bennet L, Peebles DM, Edwards AD, Rios A, Hanson MA. (1998) The cerebral hemodynamic response to asphyxia and hypoxia in the near-term fetal sheep as measured by near infrared spectroscopy. Pediatr Res, 44: 951-957.
103. Graham EM, Ruis KA, Hartman AL, Northington FJ, Fox HE. (2008) A systematic review of the role of intrapartum hypoxia-ischemia in the causation of neonatal encephalopathy. Am J Obstet Gynecol, 199: 587-595.
104. King T, Parer J. (2000) The physiology of fetal heart rate patterns and perinatal asphyxia. J Perinat Neonatal Nurs, 14: 19-39; quiz 102-103.
105. Locatelli A, Incerti M, Ghidini A, Greco M, Villa E, Paterlini G. (2008) Factors associated with umbilical artery acidemia in term infants with low Apgar scores at 5 min. Eur J Obstet Gynecol Reprod Biol, 139: 146-150.
106. Ferrari DC, Nesic O, Perez-Polo JR. (2010) Perspectives on neonatal hypoxia/ischemia-induced edema formation. Neurochem Res, 35: 1957-1965.
107. Shalak L, Perlman JM. (2004) Hypoxic-ischemic brain injury in the term infant-current concepts. Early Hum Dev, 80: 125-141.
108. Sanders RD, Manning HJ, Robertson NJ, Ma D, Edwards AD, Hagberg H, Maze M. (2010) Preconditioning and postinsult therapies for perinatal hypoxic-ischemic injury at term. Anesthesiology, 113: 233-249.
109. Hope PL, Costello AM, Cady EB, Delpy DT, Tofts PS, Chu A, Hamilton PA, Reynolds EO, Wilkie DR. (1984) Cerebral energy metabolism studied with phosphorus NMR spectroscopy in normal and birth-asphyxiated infants. Lancet, 2: 366-370.
110. Penrice J, Lorek A, Cady EB, Amess PN, Wylezinska M, Cooper CE, D'Souza P, Brown GC, Kirkbride V, Edwards AD, Wyatt JS, Reynolds EO. (1997) Proton magnetic resonance spectroscopy of the brain during acute hypoxia-ischemia and delayed cerebral energy failure in the newborn piglet. Pediatr Res, 41: 795-802.
111. Hamrick SE, Ferriero DM. (2003) The injury response in the term newborn brain: can we neuroprotect? Curr Opin Neurol, 16: 147-154.
112. Inder TE, Volpe JJ. (2000) Mechanisms of perinatal brain injury. Semin Neonatol, 5: 3-16.
113. Beal MF. (1992) Mechanisms of excitotoxicity in neurologic diseases. FASEB J, 6: 3338-3344.
114. Kristian T, Siesjo BK. (1996) Calcium-related damage in ischemia. Life Sci, 59: 357-367.
115. Jensen FE, Wang C, Stafstrom CE, Liu Z, Geary C, Stevens MC. (1998) Acute and chronic increases in excitability in rat hippocampal slices after perinatal hypoxia In vivo. J Neurophysiol, 79: 73-81.
116. Low JA. (2004) Determining the contribution of asphyxia to brain damage in the neonate. J Obstet Gynaecol Res, 30: 276-286.
117. Sajja VS, Hlavac N, VandeVord PJ. (2016) Role of Glia in Memory Deficits Following Traumatic Brain Injury: Biomarkers of Glia Dysfunction. Front Integr Neurosci, 10: 7.
118. Fleiss B, Gressens P. (2012) Tertiary mechanisms of brain damage: a new hope for treatment of cerebral palsy? Lancet Neurol, 11: 556-566.

119. Martinez-Biarge M, Diez-Sebastian J, Wusthoff CJ, Mercuri E, Cowan FM. (2013) Antepartum and intrapartum factors preceding neonatal hypoxic-ischemic encephalopathy. Pediatrics, 132: e952-959.
120. Apgar V. (1953) A proposal for a new method of evaluation of the newborn infant. Curr Res Anesth Analg, 32: 260-267.
121. Azzopardi DV, Strohm B, Edwards AD, Dyet L, Halliday HL, Juszczak E, Kapellou O, Levene M, Marlow N, Porter E, Thoresen M, Whitelaw A, Brocklehurst P, Group TS. (2009) Moderate hypothermia to treat perinatal asphyxial encephalopathy. N Engl J Med, 361: 1349-1358.
122. Low JA, Galbraith RS, Muir DW, Killen HL, Pater EA, Karchmar EJ. (1984) Factors associated with motor and cognitive deficits in children after intrapartum fetal hypoxia. Am J Obstet Gynecol, 148: 533-539.
123. Perlman JM, Tack ED, Martin T, Shackelford G, Amon E. (1989) Acute systemic organ injury in term infants after asphyxia. Am J Dis Child, 143: 617-620.
124. Van Bel F, Walther FJ. (1990) Myocardial dysfunction and cerebral blood flow velocity following birth asphyxia. Acta Paediatr Scand, 79: 756-762.
125. Alderliesten T, Nikkels PG, Benders MJ, de Vries LS, Groenendaal F. (2013) Antemortem cranial MRI compared with postmortem histopathologic examination of the brain in term infants with neonatal encephalopathy following perinatal asphyxia. Arch Dis Child Fetal Neonatal Ed, 98: F304-309.
126. Nelson KB, Dambrosia JM, Ting TY, Grether JK. (1996) Uncertain value of electronic fetal monitoring in predicting cerebral palsy. N Engl J Med, 334: 613-618.
127. Levene MI, Sands C, Grindulis H, Moore JR. (1986) Comparison of two methods of predicting outcome in perinatal asphyxia. Lancet, 1: 67-69.
128. Adamson SJ, Alessandri LM, Badawi N, Burton PR, Pemberton PJ, Stanley F. (1995) Predictors of neonatal encephalopathy in full-term infants. BMJ, 311: 598-602.
129. Fenichel GM. (1983) Hypoxic-ischemic encephalopathy in the newborn. Arch Neurol, 40: 261-266.
130. Azzopardi D, Strohm B, Marlow N, Brocklehurst P, Deierl A, Eddama O, Goodwin J, Halliday HL, Juszczak E, Kapellou O, Levene M, Linsell L, Omar O, Thoresen M, Tusor N, Whitelaw A, Edwards AD, Group TS. (2014) Effects of hypothermia for perinatal asphyxia on childhood outcomes. N Engl J Med, 371: 140-149.
131. Grigg-Damberger MM, Coker SB, Halsey CL, Anderson CL. (1989) Neonatal burst suppression: its developmental significance. Pediatr Neurol, 5: 84-92.
132. Holmes G, Rowe J, Hafford J, Schmidt R, Testa M, Zimmerman A. (1982) Prognostic value of the electroencephalogram in neonatal asphyxia. Electroencephalogr Clin Neurophysiol, 53: 60-72.
133. Monod N, Pajot N, Guidasci S. (1972) The neonatal EEG: statistical studies and prognostic value in full-term and pre-term babies. Electroencephalogr Clin Neurophysiol, 32: 529-544.
134. Watanabe K, Miyazaki S, Hara K, Hakamada S. (1980) Behavioral state cycles, background EEGs and prognosis of newborns with perinatal hypoxia. Electroencephalogr Clin Neurophysiol, 49: 618-625.
135. Connell J, Oozeer R, de Vries L, Dubowitz LM, Dubowitz V. (1989) Clinical

and EEG response to anticonvulsants in neonatal seizures. Arch Dis Child, 64: 459-464.
136. Toet MC, van der Meij W, de Vries LS, Uiterwaal CS, van Huffelen KC. (2002) Comparison between simultaneously recorded amplitude integrated electroencephalogram (cerebral function monitor) and standard electroencephalogram in neonates. Pediatrics, 109: 772-779.
137. Eken P, Toet MC, Groenendaal F, de Vries LS. (1995) Predictive value of early neuroimaging, pulsed Doppler and neurophysiology in full term infants with hypoxic-ischaemic encephalopathy. Arch Dis Child Fetal Neonatal Ed, 73: F75-80.
138. Hellstrom-Westas L, Rosen I, Svenningsen NW. (1995) Predictive value of early continuous amplitude integrated EEG recordings on outcome after severe birth asphyxia in full term infants. Arch Dis Child Fetal Neonatal Ed, 72: F34-38.
139. van Rooij LG, Toet MC, Osredkar D, van Huffelen AC, Groenendaal F, de Vries LS. (2005) Recovery of amplitude integrated electroencephalographic background patterns within 24 hours of perinatal asphyxia. Arch Dis Child Fetal Neonatal Ed, 90: F245-251.
140. Rutherford M, Pennock J, Schwieso J, Cowan F, Dubowitz L. (1996) Hypoxic-ischaemic encephalopathy: early and late magnetic resonance imaging findings in relation to outcome. Arch Dis Child Fetal Neonatal Ed, 75: F145-151.
141. Alderliesten T, de Vries LS, Benders MJ, Koopman C, Groenendaal F. (2011) MR imaging and outcome of term neonates with perinatal asphyxia: value of diffusion-weighted MR imaging and (1)H MR spectroscopy. Radiology, 261: 235-242.
142. Harteman JC, Groenendaal F, Toet MC, Benders MJ, Van Haastert IC, Nievelstein RA, Koopman-Esseboom C, de Vries LS. (2013) Diffusion-weighted imaging changes in cerebral watershed distribution following neonatal encephalopathy are not invariably associated with an adverse outcome. Dev Med Child Neurol, 55: 642-653.
143. Rutherford M, Counsell S, Allsop J, Boardman J, Kapellou O, Larkman D, Hajnal J, Edwards D, Cowan F. (2004) Diffusion-weighted magnetic resonance imaging in term perinatal brain injury: a comparison with site of lesion and time from birth. Pediatrics, 114: 1004-1014.
144. Hull J, Dodd KL. (1992) Falling incidence of hypoxic-ischaemic encephalopathy in term infants. Br J Obstet Gynaecol, 99: 386-391.
145. Agarwal R, Jain A, Deorari AK, Paul VK. (2008) Post-resuscitation management of asphyxiated neonates. Indian J Pediatr, 75: 175-180.
146. Antonucci R, Porcella A, Pilloni MD. (2014) Perinatal asphyxia in the term newborn. J pediatric neonatal individ medicine, 2014;3: e030269.
147. Saugstad OD. (2003) Oxygen Toxicity at Birth: The Pieces Are Put Together. Pediatric Research, 54: 789-789.
148. Klinger G, Beyene J, Shah P, Perlman M. (2005) Do hyperoxaemia and hypocapnia add to the risk of brain injury after intrapartum asphyxia? Arch Dis Child Fetal Neonatal Ed, 90: F49-52.
149. Spies EE, Lababidi SL, McBride MC. (2014) Early hyperglycemia is associated with poor gross motor outcome in asphyxiated term newborns. Pediatr Neurol, 50: 586-590.

150. van Rooij LG, Toet MC, van Huffelen AC, Groenendaal F, Laan W, Zecic A, de Haan T, van Straaten IL, Vrancken S, van Wezel G, van der Sluijs J, Ter Horst H, Gavilanes D, Laroche S, Naulaers G, de Vries LS. (2010) Effect of treatment of subclinical neonatal seizures detected with aEEG: randomized, controlled trial. Pediatrics, 125: e358-366.
151. Caplan MS, Hedlund E, Adler L, Hsueh W. (1994) Role of asphyxia and feeding in a neonatal rat model of necrotizing enterocolitis. Pediatr Pathol, 14: 1017-1028.
152. Sävman K. (2008) The Brain and Resuscitation. NeoReviews, 9: e513-e519.
153. Jacobs SE, Berg M, Hunt R, Tarnow-Mordi WO, Inder TE, Davis PG. (2013) Cooling for newborns with hypoxic ischaemic encephalopathy. Cochrane Database Syst Rev: CD003311.
154. Shankaran S, Pappas A, McDonald SA, Vohr BR, Hintz SR, Yolton K, Gustafson KE, Leach TM, Green C, Bara R, Petrie Huitema CM, Ehrenkranz RA, Tyson JE, Das A, Hammond J, Peralta-Carcelen M, Evans PW, Heyne RJ, Wilson-Costello DE, Vaucher YE, Bauer CR, Dusick AM, Adams-Chapman I, Goldstein RF, Guillet R, Papile LA, Higgins RD, Eunice Kennedy Shriver NNRN. (2012) Childhood outcomes after hypothermia for neonatal encephalopathy. N Engl J Med, 366: 2085-2092.
155. Thoresen M, Tooley J, Liu X, Jary S, Fleming P, Luyt K, Jain A, Cairns P, Harding D, Sabir H. (2013) Time is brain: starting therapeutic hypothermia within three hours after birth improves motor outcome in asphyxiated newborns. Neonatology, 104: 228-233.
156. Shankaran S. (2010) Neonatal Encephalopathy: Treatment With Hypothermia. NeoReviews, 11: e85-e92.
157. Carty ML, Wixey JA, Colditz PB, Buller KM. (2008) Post-insult minocycline treatment attenuates hypoxia-ischemia-induced neuroinflammation and white matter injury in the neonatal rat: a comparison of two different dose regimens. Int J Dev Neurosci, 26: 477-485.
158. Chew LJ, Takanohashi A, Bell M. (2006) Microglia and inflammation: impact on developmental brain injuries. Ment Retard Dev Disabil Res Rev, 12: 105-112.
159. McRae A, Gilland E, Bona E, Hagberg H. (1995) Microglia activation after neonatal hypoxic-ischemia. Brain Res Dev Brain Res, 84: 245-252.
160. Simao F, Matte A, Pagnussat AS, Netto CA, Salbego CG. (2012) Resveratrol preconditioning modulates inflammatory response in the rat hippocampus following global cerebral ischemia. Neurochem Int, 61: 659-665.
161. Ziemka-Nalecz M, Jaworska J, Zalewska T. (2017) Insights Into the Neuroinflammatory Responses After Neonatal Hypoxia-Ischemia. J Neuropathol Exp Neurol, 76: 644-654.
162. Juul SE, Mayock DE, Comstock BA, Heagerty PJ. (2015) Neuroprotective potential of erythropoietin in neonates; design of a randomized trial. Matern Health Neonatol Perinatol, 1: 27.
163. Lan KM, Tien LT, Cai Z, Lin S, Pang Y, Tanaka S, Rhodes PG, Bhatt AJ, Savich RD, Fan LW. (2016) Erythropoietin Ameliorates Neonatal Hypoxia-Ischemia-Induced Neurobehavioral Deficits, Neuroinflammation, and Hippocampal Injury in the Juvenile Rat. Int J Mol Sci, 17: 289.
164. Messier AM, Ohls RK. (2014) Neuroprotective effects of erythropoiesis-

stimulating agents in term and preterm neonates. Curr Opin Pediatr, 26: 139-145.
165. Rangarajan V, Juul SE. (2014) Erythropoietin: emerging role of erythropoietin in neonatal neuroprotection. Pediatr Neurol, 51: 481-488.
166. van der Kooij MA, Groenendaal F, Kavelaars A, Heijnen CJ, van Bel F. (2008) Neuroprotective properties and mechanisms of erythropoietin in in vitro and in vivo experimental models for hypoxia/ischemia. Brain Res Rev, 59: 22-33.
167. Elmahdy H, El-Mashad AR, El-Bahrawy H, El-Gohary T, El-Barbary A, Aly H. (2010) Human recombinant erythropoietin in asphyxia neonatorum: pilot trial. Pediatrics, 125: e1135-1142.
168. Benders MJ, van der Aa NE, Roks M, van Straaten HL, Isgum I, Viergever MA, Groenendaal F, de Vries LS, van Bel F. (2014) Feasibility and safety of erythropoietin for neuroprotection after perinatal arterial ischemic stroke. J Pediatr, 164: 481-486.
169. Reiter RJ, Calvo JR, Karbownik M, Qi W, Tan DX. (2000) Melatonin and its relation to the immune system and inflammation. Ann N Y Acad Sci, 917: 376-386.
170. Villapol S, Fau S, Renolleau S, Biran V, Charriaut-Marlangue C, Baud O. (2011) Melatonin promotes myelination by decreasing white matter inflammation after neonatal stroke. Pediatr Res, 69: 51-55.
171. Robertson NJ, Faulkner S, Fleiss B, Bainbridge A, Andorka C, Price D, Powell E, Lecky-Thompson L, Thei L, Chandrasekaran M, Hristova M, Cady EB, Gressens P, Golay X, Raivich G. (2013) Melatonin augments hypothermic neuroprotection in a perinatal asphyxia model. Brain, 136: 90-105.
172. Ma D, Hossain M, Chow A, Arshad M, Battson RM, Sanders RD, Mehmet H, Edwards AD, Franks NP, Maze M. (2005) Xenon and hypothermia combine to provide neuroprotection from neonatal asphyxia. Ann Neurol, 58: 182-193.
173. Franks NP, Dickinson R, de Sousa SL, Hall AC, Lieb WR. (1998) How does xenon produce anaesthesia? Nature, 396: 324.
174. Goto T, Saito H, Shinkai M, Nakata Y, Ichinose F, Morita S. (1997) Xenon provides faster emergence from anesthesia than does nitrous oxide-sevoflurane or nitrous oxide-isoflurane. Anesthesiology, 86: 1273-1278.
175. Faulkner S, Bainbridge A, Kato T, Chandrasekaran M, Kapetanakis AB, Hristova M, Liu M, Evans S, De Vita E, Kelen D, Sanders RD, Edwards AD, Maze M, Cady EB, Raivich G, Robertson NJ. (2011) Xenon augmented hypothermia reduces early lactate/N-acetylaspartate and cell death in perinatal asphyxia. Ann Neurol, 70: 133-150.
176. Jantzie LL, Todd KG. (2010) Doxycycline inhibits proinflammatory cytokines but not acute cerebral cytogenesis after hypoxia-ischemia in neonatal rats. J Psychiatry Neurosci, 35: 20-32.
177. Kim HJ, Rowe M, Ren M, Hong JS, Chen PS, Chuang DM. (2007) Histone deacetylase inhibitors exhibit anti-inflammatory and neuroprotective effects in a rat permanent ischemic model of stroke: multiple mechanisms of action. J Pharmacol Exp Ther, 321: 892-901.
178. Liu XS, Chopp M, Kassis H, Jia LF, Hozeska-Solgot A, Zhang RL, Chen C, Cui YS, Zhang ZG. (2012) Valproic acid increases white matter repair and neurogenesis after stroke. Neuroscience, 220: 313-321.
179. Ren M, Leng Y, Jeong M, Leeds PR, Chuang DM. (2004) Valproic acid reduces

brain damage induced by transient focal cerebral ischemia in rats: potential roles of histone deacetylase inhibition and heat shock protein induction. J Neurochem, 89: 1358-1367.
180. Hagberg H, Mallard C, Ferriero DM, Vannucci SJ, Levison SW, Vexler ZS, Gressens P. (2015) The role of inflammation in perinatal brain injury. Nat Rev Neurol, 11: 192-208.
181. Finer NN, Robertson CM, Richards RT, Pinnell LE, Peters KL. (1981) Hypoxic-ischemic encephalopathy in term neonates: perinatal factors and outcome. J Pediatr, 98: 112-117.
182. Vannucci RC, Perlman JM. (1997) Interventions for perinatal hypoxic-ischemic encephalopathy. Pediatrics, 100: 1004-1014.
183. Mwaniki MK, Atieno M, Lawn JE, Newton CR. (2012) Long-term neurodevelopmental outcomes after intrauterine and neonatal insults: a systematic review. Lancet, 379: 445-452.
184. van Handel M, Swaab H, de Vries LS, Jongmans MJ. (2010) Behavioral outcome in children with a history of neonatal encephalopathy following perinatal asphyxia. J Pediatr Psychol, 35: 286-295.
185. Pin TW, Eldridge B, Galea MP. (2009) A review of developmental outcomes of term infants with post-asphyxia neonatal encephalopathy. Eur J Paediatr Neurol, 13: 224-234.
186. de Vries LS, Jongmans MJ. (2010) Long-term outcome after neonatal hypoxic-ischaemic encephalopathy. Arch Dis Child Fetal Neonatal Ed, 95: F220-224.
187. Armstrong-Wells J, Bernard TJ, Boada R, Manco-Johnson M. (2010) Neurocognitive outcomes following neonatal encephalopathy. NeuroRehabilitation, 26: 27-33.
188. van Handel M, Swaab H, de Vries LS, Jongmans MJ. (2007) Long-term cognitive and behavioral consequences of neonatal encephalopathy following perinatal asphyxia: a review. Eur J Pediatr, 166: 645-654.
189. van Laerhoven H, de Haan TR, Offringa M, Post B, van der Lee JH. (2013) Prognostic tests in term neonates with hypoxic-ischemic encephalopathy: a systematic review. Pediatrics, 131: 88-98.
190. Mercuri E, Guzzetta A, Haataja L, Cowan F, Rutherford M, Counsell S, Papadimitriou M, Cioni G, Dubowitz L. (1999) Neonatal neurological examination in infants with hypoxic ischaemic encephalopathy: correlation with MRI findings. Neuropediatrics, 30: 83-89.
191. Bernson-Leung ME, Rivkin MJ. (2016) Stroke in Neonates and Children. Pediatr Rev, 37: 463-477.
192. Chalmers EA. (2005) Perinatal stroke--risk factors and management. Br J Haematol, 130: 333-343.
193. Kirton A, deVeber G. (2015) Paediatric stroke: pressing issues and promising directions. Lancet Neurol, 14: 92-102.
194. Raju TN, Nelson KB, Ferriero D, Lynch JK, Participants N-NPSW. (2007) Ischemic perinatal stroke: summary of a workshop sponsored by the National Institute of Child Health and Human Development and the National Institute of Neurological Disorders and Stroke. Pediatrics, 120: 609-616.
195. Lynch JK, Nelson KB. (2001) Epidemiology of perinatal stroke. Curr Opin Pediatr, 13: 499-505.
196. Giraud A, Guiraut C, Chevin M, Chabrier S, Sebire G. (2017) Role of Perinatal

Inflammation in Neonatal Arterial Ischemic Stroke. Front Neurol, 8: 612.
197. Lee J, Croen LA, Backstrand KH, Yoshida CK, Henning LH, Lindan C, Ferriero DM, Fullerton HJ, Barkovich AJ, Wu YW. (2005) Maternal and infant characteristics associated with perinatal arterial stroke in the infant. JAMA, 293: 723-729.
198. deVeber GA, Kirton A, Booth FA, Yager JY, Wirrell EC, Wood E, Shevell M, Surmava AM, McCusker P, Massicotte MP, MacGregor D, MacDonald EA, Meaney B, Levin S, Lemieux BG, Jardine L, Humphreys P, David M, Chan AK, Buckley DJ, Bjornson BH. (2017) Epidemiology and Outcomes of Arterial Ischemic Stroke in Children: The Canadian Pediatric Ischemic Stroke Registry. Pediatr Neurol, 69: 58-70.
199. Fitzgerald KC, Golomb MR. (2007) Neonatal arterial ischemic stroke and sinovenous thrombosis associated with meningitis. J Child Neurol, 22: 818-822.
200. Harteman JC, Groenendaal F, Kwee A, Welsing PM, Benders MJ, de Vries LS. (2012) Risk factors for perinatal arterial ischaemic stroke in full-term infants: a case-control study. Arch Dis Child Fetal Neonatal Ed, 97: F411-416.
201. Mann JR, McDermott S, Pan C, Hardin JW. (2013) Maternal hypertension and intrapartum fever are associated with increased risk of ischemic stroke during infancy. Dev Med Child Neurol, 55: 58-64.
202. Ment LR, Ehrenkranz RA, Duncan CC. (1986) Bacterial meningitis as an etiology of perinatal cerebral infarction. Pediatr Neurol, 2: 276-279.
203. Elbers J, Viero S, MacGregor D, DeVeber G, Moore AM. (2011) Placental pathology in neonatal stroke. Pediatrics, 127: e722-729.
204. Gunther G, Junker R, Strater R, Schobess R, Kurnik K, Heller C, Kosch A, Nowak-Gottl U, Childhood Stroke Study G. (2000) Symptomatic ischemic stroke in full-term neonates : role of acquired and genetic prothrombotic risk factors. Stroke, 31: 2437-2441.
205. Kenet G, Lutkhoff LK, Albisetti M, Bernard T, Bonduel M, Brandao L, Chabrier S, Chan A, deVeber G, Fiedler B, Fullerton HJ, Goldenberg NA, Grabowski E, Gunther G, Heller C, Holzhauer S, Iorio A, Journeycake J, Junker R, Kirkham FJ, Kurnik K, Lynch JK, Male C, Manco-Johnson M, Mesters R, Monagle P, van Ommen CH, Raffini L, Rostasy K, Simioni P, Strater RD, Young G, Nowak-Gottl U. (2010) Impact of thrombophilia on risk of arterial ischemic stroke or cerebral sinovenous thrombosis in neonates and children: a systematic review and meta-analysis of observational studies. Circulation, 121: 1838-1847.
206. Kirton A, Armstrong-Wells J, Chang T, Deveber G, Rivkin MJ, Hernandez M, Carpenter J, Yager JY, Lynch JK, Ferriero DM, International Pediatric Stroke Study I. (2011) Symptomatic neonatal arterial ischemic stroke: the International Pediatric Stroke Study. Pediatrics, 128: e1402-1410.
207. Kraus FT, Acheen VI. (1999) Fetal thrombotic vasculopathy in the placenta: cerebral thrombi and infarcts, coagulopathies, and cerebral palsy. Hum Pathol, 30: 759-769.
208. Hillier SL, Witkin SS, Krohn MA, Watts DH, Kiviat NB, Eschenbach DA. (1993) The relationship of amniotic fluid cytokines and preterm delivery, amniotic fluid infection, histologic chorioamnionitis, and chorioamnion infection. Obstet Gynecol, 81: 941-948.

209. Hubel CA. (1999) Oxidative stress in the pathogenesis of preeclampsia. Proc Soc Exp Biol Med, 222: 222-235.
210. Walker JJ. (1998) Antioxidants and inflammatory cell response in preeclampsia. Semin Reprod Endocrinol, 16: 47-55.
211. Novales JS, Salva AM, Modanlou HD, Kaplan DL, Delcastillo J, Andresen J, Medlock ES. (1993) Maternal Administration of Granulocyte Colony-Stimulating Factor Improves Neonatal Rat Survival after a Lethal Group-B Streptococcal Infection. Blood, 81: 923-927.
212. Shalak LF, Laptook AR, Jafri HS, Ramilo O, Perlman JM. (2002) Clinical chorioamnionitis, elevated cytokines, and brain injury in term infants. Pediatrics, 110: 673-680.
213. Crocker IP, Tansinda DM, Baker PN. (2004) Altered cell kinetics in cultured placental villous explants in pregnancies complicated by pre-eclampsia and intrauterine growth restriction. J Pathol, 204: 11-18.
214. Guiraut C, Cauchon N, Lepage M, Sebire G. (2016) Perinatal Arterial Ischemic Stroke Is Associated to Materno-Fetal Immune Activation and Intracranial Arteritis. Int J Mol Sci, 17: 1980.
215. Nelson KB, Lynch JK. (2004) Stroke in newborn infants. Lancet Neurol, 3: 150-158.
216. Osborn AG. (1999) Diagnostic Cerebral Angiography. American Journal of Neuroradiology, 20: 1767-1769.
217. Miller V. (2000) Neonatal cerebral infarction. Semin Pediatr Neurol, 7: 278-288.
218. Husson B, Hertz-Pannier L, Adamsbaum C, Renaud C, Presles E, Dinomais M, Kossorotoff M, Landrieu P, Chabrier S. (2016) MR angiography findings in infants with neonatal arterial ischemic stroke in the middle cerebral artery territory: A prospective study using circle of Willis MR angiography. Eur J Radiol, 85: 1329-1335.
219. Larouche A, Roy M, Kadhim H, Tsanaclis AM, Fortin D, Sebire G. (2005) Neuronal injuries induced by perinatal hypoxic-ischemic insults are potentiated by prenatal exposure to lipopolysaccharide: animal model for perinatally acquired encephalopathy. Dev Neurosci, 27: 134-142.
220. Mallard C, Hagberg H. (2007) Inflammation-induced preconditioning in the immature brain. Semin Fetal Neonatal Med, 12: 280-286.
221. Wang X, Hagberg H, Nie C, Zhu C, Ikeda T, Mallard C. (2007) Dual role of intrauterine immune challenge on neonatal and adult brain vulnerability to hypoxia-ischemia. J Neuropathol Exp Neurol, 66: 552-561.
222. Davidson JO, Wassink G, van den Heuij LG, Bennet L, Gunn AJ. (2015) Therapeutic Hypothermia for Neonatal Hypoxic-Ischemic Encephalopathy - Where to from Here? Front Neurol, 6: 198.
223. Kratzer I, Chip S, Vexler ZS. (2014) Barrier mechanisms in neonatal stroke. Front Neurosci, 8: 359.
224. Wang Q, Tang XN, Yenari MA. (2007) The inflammatory response in stroke. J Neuroimmunol, 184: 53-68.
225. Eliwan HO, Watson RW, Aslam S, Regan I, Philbin B, O'Hare FM, O'Neill A, Preston R, Blanco A, Grant T, Nolan B, Smith O, Molloy EJ. (2015) Neonatal brain injury and systemic inflammation: modulation by activated protein C ex vivo. Clin Exp Immunol, 179: 477-484.

226. Savard A, Brochu ME, Chevin M, Guiraut C, Grbic D, Sebire G. (2015) Neuronal self-injury mediated by IL-1beta and MMP-9 in a cerebral palsy model of severe neonatal encephalopathy induced by immune activation plus hypoxia-ischemia. J Neuroinflammation, 12: 111.
227. Savard A, Lavoie K, Brochu ME, Grbic D, Lepage M, Gris D, Sebire G. (2013) Involvement of neuronal IL-1beta in acquired brain lesions in a rat model of neonatal encephalopathy. J Neuroinflammation, 10: 110.
228. Stridh L, Smith PL, Naylor AS, Wang X, Mallard C. (2011) Regulation of toll-like receptor 1 and -2 in neonatal mice brains after hypoxia-ischemia. J Neuroinflammation, 8: 45.
229. Wang X, Stridh L, Li W, Dean J, Elmgren A, Gan L, Eriksson K, Hagberg H, Mallard C. (2009) Lipopolysaccharide sensitizes neonatal hypoxic-ischemic brain injury in a MyD88-dependent manner. J Immunol, 183: 7471-7477.
230. Schouten M, Wiersinga WJ, Levi M, van der Poll T. (2008) Inflammation, endothelium, and coagulation in sepsis. J Leukoc Biol, 83: 536-545.
231. Gelderblom M, Leypoldt F, Steinbach K, Behrens D, Choe CU, Siler DA, Arumugam TV, Orthey E, Gerloff C, Tolosa E, Magnus T. (2009) Temporal and spatial dynamics of cerebral immune cell accumulation in stroke. Stroke, 40: 1849-1857.
232. Liesz A, Zhou W, Mracsko E, Karcher S, Bauer H, Schwarting S, Sun L, Bruder D, Stegemann S, Cerwenka A, Sommer C, Dalpke AH, Veltkamp R. (2011) Inhibition of lymphocyte trafficking shields the brain against deleterious neuroinflammation after stroke. Brain, 134: 704-720.
233. Bajnok A, Berta L, Orban C, Tulassay T, Toldi G. (2018) Cytokine production pattern of T lymphocytes in neonatal arterial ischemic stroke during the first month of life-a case study. J Neuroinflammation, 15: 191.
234. Vogelgesang A, Becker KJ, Dressel A. (2014) Immunological consequences of ischemic stroke. Acta Neurol Scand, 129: 1-12.
235. Yilmaz G, Arumugam TV, Stokes KY, Granger DN. (2006) Role of T lymphocytes and interferon-gamma in ischemic stroke. Circulation, 113: 2105-2112.
236. Yururer D, Teber S, Deda G, Egin Y, Akar N. (2009) The relation between cytokines, soluble endothelial protein C receptor, and factor VIII levels in Turkish pediatric stroke patients. Clin Appl Thromb Hemost, 15: 545-551.
237. Siniscalchi A, Gallelli L, Malferrari G, Pirritano D, Serra R, Santangelo E, De Sarro G. (2014) Cerebral stroke injury: the role of cytokines and brain inflammation. J Basic Clin Physiol Pharmacol, 25: 131-137.
238. Spera PA, Ellison JA, Feuerstein GZ, Barone FC. (1998) IL-10 reduces rat brain injury following focal stroke. Neurosci Lett, 251: 189-192.
239. Zhu Y, Yang GY, Ahlemeyer B, Pang L, Che XM, Culmsee C, Klumpp S, Krieglstein J. (2002) Transforming growth factor-beta 1 increases bad phosphorylation and protects neurons against damage. J Neurosci, 22: 3898-3909.
240. Carlsson Y, Schwendimann L, Vontell R, Rousset CI, Wang X, Lebon S, Charriaut-Marlangue C, Supramaniam V, Hagberg H, Gressens P, Jacotot E. (2011) Genetic inhibition of caspase-2 reduces hypoxic-ischemic and excitotoxic neonatal brain injury. Ann Neurol, 70: 781-789.
241. Nijboer CH, Heijnen CJ, van der Kooij MA, Zijlstra J, van Velthoven CT,

Culmsee C, van Bel F, Hagberg H, Kavelaars A. (2011) Targeting the p53 pathway to protect the neonatal ischemic brain. Ann Neurol, 70: 255-264.
242. Estan J, Hope P. (1997) Unilateral neonatal cerebral infarction in full term infants. Arch Dis Child Fetal Neonatal Ed, 76: F88-93.
243. Levy SR, Abroms IF, Marshall PC, Rosquete EE. (1985) Seizures and cerebral infarction in the full-term newborn. Ann Neurol, 17: 366-370.
244. Ramaswamy V, Miller SP, Barkovich AJ, Partridge JC, Ferriero DM. (2004) Perinatal stroke in term infants with neonatal encephalopathy. Neurology, 62: 2088-2091.
245. Gancia P, Pomero G. (2012) Therapeutic hypothermia in the prevention of hypoxic-ischaemic encephalopathy: new categories to be enrolled. J Matern Fetal Neonatal Med, 25 Suppl 4: 94-96.
246. Roka A, Beko G, Halasz J, Toldi G, Lakatos P, Azzopardi D, Tulassay T, Szabo M. (2013) Changes in serum cytokine and cortisol levels in normothermic and hypothermic term neonates after perinatal asphyxia. Inflamm Res, 62: 81-87.
247. Harbert MJ, Tam EW, Glass HC, Bonifacio SL, Haeusslein LA, Barkovich AJ, Jeremy RJ, Rogers EE, Glidden DV, Ferriero DM. (2011) Hypothermia is correlated with seizure absence in perinatal stroke. J Child Neurol, 26: 1126-1130.
248. Mercuri E, Rutherford M, Cowan F, Pennock J, Counsell S, Papadimitriou M, Azzopardi D, Bydder G, Dubowitz L. (1999) Early prognostic indicators of outcome in infants with neonatal cerebral infarction: a clinical, electroencephalogram, and magnetic resonance imaging study. Pediatrics, 103: 39-46.
249. Golomb MR, MacGregor DL, Domi T, Armstrong DC, McCrindle BW, Mayank S, deVeber GA. (2001) Presumed pre- or perinatal arterial ischemic stroke: risk factors and outcomes. Ann Neurol, 50: 163-168.
250. McLinden A, Baird AD, Westmacott R, Anderson PE, deVeber G. (2007) Early cognitive outcome after neonatal stroke. J Child Neurol, 22: 1111-1116.
251. Westmacott R, Askalan R, MacGregor D, Anderson P, Deveber G. (2010) Cognitive outcome following unilateral arterial ischaemic stroke in childhood: effects of age at stroke and lesion location. Dev Med Child Neurol, 52: 386-393.
252. Anderson V, Catroppa C, Morse S, Haritou F, Rosenfeld J. (2005) Functional plasticity or vulnerability after early brain injury? Pediatrics, 116: 1374-1382.
253. Ferriero DM. (2016) The Vulnerable Newborn Brain: Imaging Patterns of Acquired Perinatal Injury. Neonatology, 109: 345-351.
254. Riva D, Cazzaniga L. (1986) Late Effects of Unilateral Brain-Lesions Sustained before and after Age One. Neuropsychologia, 24: 423-428.
255. Offner H, Subramanian S, Parker SM, Afentoulis ME, Vandenbark AA, Hurn PD. (2006) Experimental stroke induces massive, rapid activation of the peripheral immune system. J Cereb Blood Flow Metab, 26: 654-665.
256. Okazaki K, Nishida A, Kato M, Kozawa K, Uga N, Kimura H. (2006) Elevation of cytokine concentrations in asphyxiated neonates. Biol Neonate, 89: 183-189.
257. Liu F, McCullough LD. (2013) Inflammatory responses in hypoxic ischemic encephalopathy. Acta Pharmacol Sin, 34: 1121-1130.
258. Algra SO, Groeneveld KM, Schadenberg AW, Haas F, Evens FC, Meerding J,

Koenderman L, Jansen NJ, Prakken BJ. (2013) Cerebral ischemia initiates an immediate innate immune response in neonates during cardiac surgery. J Neuroinflammation, 10: 24.
259. Dommergues MA, Plaisant F, Verney C, Gressens P. (2003) Early microglial activation following neonatal excitotoxic brain damage in mice: a potential target for neuroprotection. Neuroscience, 121: 619-628.
260. Bona E, Andersson AL, Blomgren K, Gilland E, Puka-Sundvall M, Gustafson K, Hagberg H. (1999) Chemokine and inflammatory cell response to hypoxia-ischemia in immature rats. Pediatr Res, 45: 500-509.
261. Hagberg H, Gressens P, Mallard C. (2012) Inflammation during fetal and neonatal life: implications for neurologic and neuropsychiatric disease in children and adults. Ann Neurol, 71: 444-457.
262. Zheng Z, Yenari MA. (2004) Post-ischemic inflammation: molecular mechanisms and therapeutic implications. Neurol Res, 26: 884-892.
263. Yong VW, Marks S. (2010) The interplay between the immune and central nervous systems in neuronal injury. Neurology, 74 Suppl 1: S9-S16.
264. Favrais G, van de Looij Y, Fleiss B, Ramanantsoa N, Bonnin P, Stoltenburg-Didinger G, Lacaud A, Saliba E, Dammann O, Gallego J, Sizonenko S, Hagberg H, Lelievre V, Gressens P. (2011) Systemic inflammation disrupts the developmental program of white matter. Ann Neurol, 70: 550-565.
265. Leviton A, Gressens P. (2007) Neuronal damage accompanies perinatal white-matter damage. Trends Neurosci, 30: 473-478.
266. Nagamoto-Combs K, McNeal DW, Morecraft RJ, Combs CK. (2007) Prolonged microgliosis in the rhesus monkey central nervous system after traumatic brain injury. J Neurotrauma, 24: 1719-1742.
267. Ramlackhansingh AF, Brooks DJ, Greenwood RJ, Bose SK, Turkheimer FE, Kinnunen KM, Gentleman S, Heckemann RA, Gunanayagam K, Gelosa G, Sharp DJ. (2011) Inflammation after trauma: microglial activation and traumatic brain injury. Ann Neurol, 70: 374-383.
268. Winerdal M, Winerdal ME, Kinn J, Urmaliya V, Winqvist O, Aden U. (2012) Long lasting local and systemic inflammation after cerebral hypoxic ischemia in newborn mice. PLoS One, 7: e36422.
269. Jakovcevski I, Wu J, Karl N, Leshchyns'ka I, Sytnyk V, Chen J, Irintchev A, Schachner M. (2007) Glial scar expression of CHL1, the close homolog of the adhesion molecule L1, limits recovery after spinal cord injury. J Neurosci, 27: 7222-7233.
270. Sofroniew MV. (2009) Molecular dissection of reactive astrogliosis and glial scar formation. Trends Neurosci, 32: 638-647.
271. Tang X, Davies JE, Davies SJ. (2003) Changes in distribution, cell associations, and protein expression levels of NG2, neurocan, phosphacan, brevican, versican V2, and tenascin-C during acute to chronic maturation of spinal cord scar tissue. J Neurosci Res, 71: 427-444.
272. Bruhn T, Levy LM, Nielsen M, Christensen T, Johansen FF, Diemer NH. (2000) Ischemia induced changes in expression of the astrocyte glutamate transporter GLT1 in hippocampus of the rat. Neurochem Int, 37: 277-285.
273. Ozanne SE, Constancia M. (2007) Mechanisms of disease: the developmental origins of disease and the role of the epigenotype. Nat Clin Pract Endocrinol Metab, 3: 539-546.

274. Peleg S, Sananbenesi F, Zovoilis A, Burkhardt S, Bahari-Javan S, Agis-Balboa RC, Cota P, Wittnam JL, Gogol-Doering A, Opitz L, Salinas-Riester G, Dettenhofer M, Kang H, Farinelli L, Chen W, Fischer A. (2010) Altered histone acetylation is associated with age-dependent memory impairment in mice. Science, 328: 753-756.
275. Shen S, Liu A, Li J, Wolubah C, Casaccia-Bonnefil P. (2008) Epigenetic memory loss in aging oligodendrocytes in the corpus callosum. Neurobiol Aging, 29: 452-463.
276. Galic MA, Riazi K, Heida JG, Mouihate A, Fournier NM, Spencer SJ, Kalynchuk LE, Teskey GC, Pittman QJ. (2008) Postnatal inflammation increases seizure susceptibility in adult rats. J Neurosci, 28: 6904-6913.
277. Williamson LL, Sholar PW, Mistry RS, Smith SH, Bilbo SD. (2011) Microglia and memory: modulation by early-life infection. J Neurosci, 31: 15511-15521.
278. Bilbo SD. (2010) Early-life infection is a vulnerability factor for aging-related glial alterations and cognitive decline. Neurobiol Learn Mem, 94: 57-64.
279. Boisse L, Mouihate A, Ellis S, Pittman QJ. (2004) Long-term alterations in neuroimmune responses after neonatal exposure to lipopolysaccharide. J Neurosci, 24: 4928-4934.
280. Ellis S, Mouihate A, Pittman QJ. (2006) Neonatal programming of the rat neuroimmune response: stimulus specific changes elicited by bacterial and viral mimetics. J Physiol, 571: 695-701.
281. Kerschensteiner M, Meinl E, Hohlfeld R. (2009) Neuro-immune crosstalk in CNS diseases. Neuroscience, 158: 1122-1132.
282. Schwartz M, Moalem G, Leibowitz-Amit R, Cohen IR. (1999) Innate and adaptive immune responses can be beneficial for CNS repair. Trends Neurosci, 22: 295-299.
283. Bieber AJ, Kerr S, Rodriguez M. (2003) Efficient central nervous system remyelination requires T cells. Ann Neurol, 53: 680-684.
284. Bajnok A, Berta L, Orban C, Veres G, Zadori D, Barta H, Meder U, Vecsei L, Tulassay T, Szabo M, Toldi G. (2017) Distinct cytokine patterns may regulate the severity of neonatal asphyxia-an observational study. J Neuroinflammation, 14: 244.
285. Ginhoux F, Lim S, Hoeffel G, Low D, Huber T. (2013) Origin and differentiation of microglia. Front Cell Neurosci, 7: 45.
286. Stoll G, Jander S. (1999) The role of microglia and macrophages in the pathophysiology of the CNS. Prog Neurobiol, 58: 233-247.
287. Davalos D, Grutzendler J, Yang G, Kim JV, Zuo Y, Jung S, Littman DR, Dustin ML, Gan WB. (2005) ATP mediates rapid microglial response to local brain injury in vivo. Nat Neurosci, 8: 752-758.
288. Nimmerjahn A, Kirchhoff F, Helmchen F. (2005) Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. Science, 308: 1314-1318.
289. Iadecola C, Anrather J. (2011) The immunology of stroke: from mechanisms to translation. Nat Med, 17: 796-808.
290. Jimenez S, Baglietto-Vargas D, Caballero C, Moreno-Gonzalez I, Torres M, Sanchez-Varo R, Ruano D, Vizuete M, Gutierrez A, Vitorica J. (2008) Inflammatory response in the hippocampus of PS1M146L/APP751SL mouse model of Alzheimer's disease: age-dependent switch in the microglial

phenotype from alternative to classic. J Neurosci, 28: 11650-11661.
291. Varnum MM, Ikezu T. (2012) The classification of microglial activation phenotypes on neurodegeneration and regeneration in Alzheimer's disease brain. Arch Immunol Ther Exp (Warsz), 60: 251-266.
292. Hu X, Li P, Guo Y, Wang H, Leak RK, Chen S, Gao Y, Chen J. (2012) Microglia/macrophage polarization dynamics reveal novel mechanism of injury expansion after focal cerebral ischemia. Stroke, 43: 3063-3070.
293. Del Bigio MR, Becker LE. (1994) Microglial aggregation in the dentate gyrus: a marker of mild hypoxic-ischaemic brain insult in human infants. Neuropathol Appl Neurobiol, 20: 144-151.
294. Jellema RK, Lima Passos V, Zwanenburg A, Ophelders DR, De Munter S, Vanderlocht J, Germeraad WT, Kuypers E, Collins JJ, Cleutjens JP, Jennekens W, Gavilanes AW, Seehase M, Vles HJ, Steinbusch H, Andriessen P, Wolfs TG, Kramer BW. (2013) Cerebral inflammation and mobilization of the peripheral immune system following global hypoxia-ischemia in preterm sheep. J Neuroinflammation, 10: 13.
295. Ivacko JA, Sun R, Silverstein FS. (1996) Hypoxic-ischemic brain injury induces an acute microglial reaction in perinatal rats. Pediatr Res, 39: 39-47.
296. Kaur C, Rathnasamy G, Ling EA. (2013) Roles of activated microglia in hypoxia induced neuroinflammation in the developing brain and the retina. J Neuroimmune Pharmacol, 8: 66-78.
297. Jaworska J, Ziemka-Nalecz M, Sypecka J, Zalewska T. (2017) The potential neuroprotective role of a histone deacetylase inhibitor, sodium butyrate, after neonatal hypoxia-ischemia. J Neuroinflammation, 14: 34.
298. Sen E, Levison SW. (2006) Astrocytes and developmental white matter disorders. Ment Retard Dev Disabil Res Rev, 12: 97-104.
299. Sullivan SM, Bjorkman ST, Miller SM, Colditz PB, Pow DV. (2010) Morphological changes in white matter astrocytes in response to hypoxia/ischemia in the neonatal pig. Brain Res, 1319: 164-174.
300. Roessmann U, Gambetti P. (1986) Pathological reaction of astrocytes in perinatal brain injury. Immunohistochemical study. Acta Neuropathol, 70: 302-307.
301. Monje ML, Toda H, Palmer TD. (2003) Inflammatory blockade restores adult hippocampal neurogenesis. Science, 302: 1760-1765.
302. Stoll G, Jander S, Schroeter M. (1998) Inflammation and glial responses in ischemic brain lesions. Prog Neurobiol, 56: 149-171.
303. Kim JS. (1996) Cytokines and adhesion molecules in stroke and related diseases. J Neurol Sci, 137: 69-78.
304. Sofroniew MV. (2000) Astrocyte failure as a cause of CNS dysfunction. Mol Psychiatry, 5: 230-232.
305. Miller JT, Bartley JH, Wimborne HJ, Walker AL, Hess DC, Hill WD, Carroll JE. (2005) The neuroblast and angioblast chemotaxic factor SDF-1 (CXCL12) expression is briefly up regulated by reactive astrocytes in brain following neonatal hypoxic-ischemic injury. BMC Neurosci, 6: 63.
306. Bambrick L, Kristian T, Fiskum G. (2004) Astrocyte mitochondrial mechanisms of ischemic brain injury and neuroprotection. Neurochem Res, 29: 601-608.
307. Sims NR, Nilsson M, Muyderman H. (2004) Mitochondrial glutathione: a

modulator of brain cell death. J Bioenerg Biomembr, 36: 329-333.
308. Swanson RA, Ying W, Kauppinen TM. (2004) Astrocyte influences on ischemic neuronal death. Curr Mol Med, 4: 193-205.
309. Anderson CM, Swanson RA. (2000) Astrocyte glutamate transport: review of properties, regulation, and physiological functions. Glia, 32: 1-14.
310. Romera C, Hurtado O, Botella SH, Lizasoain I, Cardenas A, Fernandez-Tome P, Leza JC, Lorenzo P, Moro MA. (2004) In vitro ischemic tolerance involves upregulation of glutamate transport partly mediated by the TACE/ADAM17-tumor necrosis factor-alpha pathway. J Neurosci, 24: 1350-1357.
311. Schousboe A, Waagepetersen HS. (2006) Glial modulation of GABAergic and glutamat ergic neurotransmission. Curr Top Med Chem, 6: 929-934.
312. Stanimirovic DB, Ball R, Durkin JP. (1997) Glutamate uptake and Na,K-ATPase activity in rat astrocyte cultures exposed to ischemia. Acta Neurochir Suppl, 70: 1-3.
313. Walz W. (2000) Role of astrocytes in the clearance of excess extracellular potassium. Neurochem Int, 36: 291-300.
314. Rossi DJ, Brady JD, Mohr C. (2007) Astrocyte metabolism and signaling during brain ischemia. Nat Neurosci, 10: 1377-1386.
315. Takano T, Oberheim N, Cotrina ML, Nedergaard M. (2009) Astrocytes and ischemic injury. Stroke, 40: S8-12.
316. Gabel S, Koncina E, Dorban G, Heurtaux T, Birck C, Glaab E, Michelucci A, Heuschling P, Grandbarbe L. (2016) Inflammation Promotes a Conversion of Astrocytes into Neural Progenitor Cells via NF-kappaB Activation. Mol Neurobiol, 53: 5041-5055.
317. Garcia JH, Liu KF, Yoshida Y, Lian J, Chen S, del Zoppo GJ. (1994) Influx of leukocytes and platelets in an evolving brain infarct (Wistar rat). Am J Pathol, 144: 188-199.
318. Shiga Y, Onodera H, Kogure K, Yamasaki Y, Yashima Y, Syozuhara H, Sendo F. (1991) Neutrophil as a mediator of ischemic edema formation in the brain. Neurosci Lett, 125: 110-112.
319. Anderson DC, Hughes BJ, Smith CW. (1981) Abnormal mobility of neonatal polymorphonuclear leukocytes. Relationship to impaired redistribution of surface adhesion sites by chemotactic factor or colchicine. J Clin Invest, 68: 863-874.
320. Anderson DC, Rothlein R, Marlin SD, Krater SS, Smith CW. (1990) Impaired transendothelial migration by neonatal neutrophils: abnormalities of Mac-1 (CD11b/CD18)-dependent adherence reactions. Blood, 76: 2613-2621.
321. Hudome S, Palmer C, Roberts RL, Mauger D, Housman C, Towfighi J. (1997) The role of neutrophils in the production of hypoxic-ischemic brain injury in the neonatal rat. Pediatr Res, 41: 607-616.
322. Palmer C, Roberts RL, Young PI. (2004) Timing of neutrophil depletion influences long-term neuroprotection in neonatal rat hypoxic-ischemic brain injury. Pediatr Res, 55: 549-556.
323. Brait VH, Jackman KA, Walduck AK, Selemidis S, Diep H, Mast AE, Guida E, Broughton BR, Drummond GR, Sobey CG. (2010) Mechanisms contributing to cerebral infarct size after stroke: gender, reperfusion, T lymphocytes, and Nox2-derived superoxide. J Cereb Blood Flow Metab, 30: 1306-1317.

324. Jander S, Kraemer M, Schroeter M, Witte OW, Stoll G. (1995) Lymphocytic infiltration and expression of intercellular adhesion molecule-1 in photochemically induced ischemia of the rat cortex. J Cereb Blood Flow Metab, 15: 42-51.
325. Northington FJ, Ferriero DM, Flock DL, Martin LJ. (2001) Delayed neurodegeneration in neonatal rat thalamus after hypoxia-ischemia is apoptosis. J Neurosci, 21: 1931-1938.
326. Albertsson AM, Bi D, Duan L, Zhang X, Leavenworth JW, Qiao L, Zhu C, Cardell S, Cantor H, Hagberg H, Mallard C, Wang X. (2014) The immune response after hypoxia-ischemia in a mouse model of preterm brain injury. J Neuroinflammation, 11: 153.
327. Ellison VJ, Mocatta TJ, Winterbourn CC, Darlow BA, Volpe JJ, Inder TE. (2005) The relationship of CSF and plasma cytokine levels to cerebral white matter injury in the premature newborn. Pediatr Res, 57: 282-286.
328. Folkerth RD, Keefe RJ, Haynes RL, Trachtenberg FL, Volpe JJ, Kinney HC. (2004) Interferon-gamma expression in periventricular leukomalacia in the human brain. Brain Pathol, 14: 265-274.
329. Yoon BH, Romero R, Kim CJ, Koo JN, Choe G, Syn HC, Chi JG. (1997) High expression of tumor necrosis factor-alpha and interleukin-6 in periventricular leukomalacia. Am J Obstet Gynecol, 177: 406-411.
330. Rostami A, Ciric B. (2013) Role of Th17 cells in the pathogenesis of CNS inflammatory demyelination. J Neurol Sci, 333: 76-87.
331. Kendall GS, Hristova M, Horn S, Dafou D, Acosta-Saltos A, Almolda B, Zbarsky V, Rumajogee P, Heuer H, Castellano B, Pfeffer K, Nedospasov SA, Peebles DM, Raivich G. (2011) TNF gene cluster deletion abolishes lipopolysaccharide-mediated sensitization of the neonatal brain to hypoxic ischemic insult. Lab Invest, 91: 328-341.
332. Vogelgesang A, May VE, Grunwald U, Bakkeboe M, Langner S, Wallaschofski H, Kessler C, Broker BM, Dressel A. (2010) Functional status of peripheral blood T-cells in ischemic stroke patients. PLoS One, 5: e8718.
333. Benjelloun N, Renolleau S, Represa A, Ben-Ari Y, Charriaut-Marlangue C. (1999) Inflammatory responses in the cerebral cortex after ischemia in the P7 neonatal Rat. Stroke, 30: 1916-1923.
334. Giuliani F, Goodyer CG, Antel JP, Yong VW. (2003) Vulnerability of human neurons to T cell-mediated cytotoxicity. J Immunol, 171: 368-379.
335. Medana I, Li Z, Flugel A, Tschopp J, Wekerle H, Neumann H. (2001) Fas ligand (CD95L) protects neurons against perforin-mediated T lymphocyte cytotoxicity. J Immunol, 167: 674-681.
336. Herz J, Koster C, Crasmoller M, Abberger H, Hansen W, Felderhoff-Muser U, Bendix I. (2018) Peripheral T Cell Depletion by FTY720 Exacerbates Hypoxic-Ischemic Brain Injury in Neonatal Mice. Front Immunol, 9: 1696.
337. Liesz A, Suri-Payer E, Veltkamp C, Doerr H, Sommer C, Rivest S, Giese T, Veltkamp R. (2009) Regulatory T cells are key cerebroprotective immunomodulators in acute experimental stroke. Nat Med, 15: 192-199.
338. del Zoppo G, Ginis I, Hallenbeck JM, Iadecola C, Wang X, Feuerstein GZ. (2000) Inflammation and stroke: putative role for cytokines, adhesion molecules and iNOS in brain response to ischemia. Brain Pathol, 10: 95-112.
339. Issekutz TB. (1991) Inhibition of in vivo lymphocyte migration to

inflammation and homing to lymphoid tissues by the TA-2 monoclonal antibody. A likely role for VLA-4 in vivo. J Immunol, 147: 4178-4184.
340. Baron JL, Madri JA, Ruddle NH, Hashim G, Janeway CA, Jr. (1993) Surface expression of alpha 4 integrin by CD4 T cells is required for their entry into brain parenchyma. J Exp Med, 177: 57-68.
341. Yednock TA, Cannon C, Fritz LC, Sanchez-Madrid F, Steinman L, Karin N. (1992) Prevention of experimental autoimmune encephalomyelitis by antibodies against alpha 4 beta 1 integrin. Nature, 356: 63-66.
342. Polman CH, O'Connor PW, Havrdova E, Hutchinson M, Kappos L, Miller DH, Phillips JT, Lublin FD, Giovannoni G, Wajgt A, Toal M, Lynn F, Panzara MA, Sandrock AW, Investigators A. (2006) A randomized, placebo-controlled trial of natalizumab for relapsing multiple sclerosis. N Engl J Med, 354: 899-910.
343. Becker K, Kindrick D, Relton J, Harlan J, Winn R. (2001) Antibody to the alpha4 integrin decreases infarct size in transient focal cerebral ischemia in rats. Stroke, 32: 206-211.
344. Lee SJ, Benveniste EN. (1999) Adhesion molecule expression and regulation on cells of the central nervous system. J Neuroimmunol, 98: 77-88.
345. Arvin B, Neville LF, Barone FC, Feuerstein GZ. (1996) The role of inflammation and cytokines in brain injury. Neurosci Biobehav Rev, 20: 445-452.
346. Chalak LF, Sanchez PJ, Adams-Huet B, Laptook AR, Heyne RJ, Rosenfeld CR. (2014) Biomarkers for severity of neonatal hypoxic-ischemic encephalopathy and outcomes in newborns receiving hypothermia therapy. J Pediatr, 164: 468-474 e461.
347. Dammann O, O'Shea TM. (2008) Cytokines and perinatal brain damage. Clin Perinatol, 35: 643-663.
348. Volpe JJ. (2001) Perinatal brain injury: from pathogenesis to neuroprotection. Ment Retard Dev Disabil Res Rev, 7: 56-64.
349. Wang X, Feuerstein GZ. (2000) Role of immune and inflammatory mediators in CNS injury. Drug News Perspect, 13: 133-140.
350. Rocha-Ferreira E, Hristova M. (2015) Antimicrobial peptides and complement in neonatal hypoxia-ischemia induced brain damage. Front Immunol, 6: 56.
351. D'Souza S, Alinauskas K, McCrea E, Goodyer C, Antel JP. (1995) Differential susceptibility of human CNS-derived cell populations to TNF-dependent and independent immune-mediated injury. J Neurosci, 15: 7293-7300.
352. Hisahara S, Shoji S, Okano H, Miura M. (1997) ICE/CED-3 family executes oligodendrocyte apoptosis by tumor necrosis factor. J Neurochem, 69: 10-20.
353. Torres C, Aranguez I, Rubio N. (1995) Expression of interferon-gamma receptors on murine oligodendrocytes and its regulation by cytokines and mitogens. Immunology, 86: 250-255.
354. Aly H, Khashaba MT, El-Ayouty M, El-Sayed O, Hasanein BM. (2006) IL-1beta, IL-6 and TNF-alpha and outcomes of neonatal hypoxic ischemic encephalopathy. Brain Dev, 28: 178-182.
355. Saliba E, Henrot A. (2001) Inflammatory mediators and neonatal brain damage. Biol Neonate, 79: 224-227.
356. Savman K, Blennow M, Gustafson K, Tarkowski E, Hagberg H. (1998) Cytokine response in cerebrospinal fluid after birth asphyxia. Pediatr Res, 43: 746-751.

357. Woiciechowsky C, Schoning B, Stoltenburg-Didinger G, Stockhammer F, Volk HD. (2004) Brain-IL-1 beta triggers astrogliosis through induction of IL-6: inhibition by propranolol and IL-10. Med Sci Monit, 10: 325-330.
358. Ziv Y, Ron N, Butovsky O, Landa G, Sudai E, Greenberg N, Cohen H, Kipnis J, Schwartz M. (2006) Immune cells contribute to the maintenance of neurogenesis and spatial learning abilities in adulthood. Nat Neurosci, 9: 268-275.
359. Ziv Y, Schwartz M. (2008) Immune-based regulation of adult neurogenesis: implications for learning and memory. Brain Behav Immun, 22: 167-176.
360. Gehrmann J, Banati RB, Wiessner C, Hossmann KA, Kreutzberg GW. (1995) Reactive microglia in cerebral ischaemia: an early mediator of tissue damage? Neuropathol Appl Neurobiol, 21: 277-289.
361. Buttini M, Boddeke H. (1995) Peripheral lipopolysaccharide stimulation induces interleukin-1 beta messenger RNA in rat brain microglial cells. Neuroscience, 65: 523-530.
362. Deng Y, Lu J, Sivakumar V, Ling EA, Kaur C. (2008) Amoeboid microglia in the periventricular white matter induce oligodendrocyte damage through expression of proinflammatory cytokines via MAP kinase signaling pathway in hypoxic neonatal rats. Brain Pathol, 18: 387-400.
363. Fan LW, Mitchell HJ, Tien LT, Rhodes PG, Cai Z. (2009) Interleukin-1beta-induced brain injury in the neonatal rat can be ameliorated by alpha-phenyl-n-tert-butyl-nitrone. Exp Neurol, 220: 143-153.
364. Liu J, Feng ZC. (2010) Increased umbilical cord plasma interleukin-1 beta levels was correlated with adverse outcomes of neonatal hypoxic-ischemic encephalopathy. J Trop Pediatr, 56: 178-182.
365. Long-Smith CM, Collins L, Toulouse A, Sullivan AM, Nolan YM. (2010) Interleukin-1beta contributes to dopaminergic neuronal death induced by lipopolysaccharide-stimulated rat glia in vitro. J Neuroimmunol, 226: 20-26.
366. Ren K, Torres R. (2009) Role of interleukin-1beta during pain and inflammation. Brain Res Rev, 60: 57-64.
367. Yabuuchi K, Minami M, Katsumata S, Yamazaki A, Satoh M. (1994) An in situ hybridization study on interleukin-1 beta mRNA induced by transient forebrain ischemia in the rat brain. Brain Res Mol Brain Res, 26: 135-142.
368. Yamasaki Y, Matsuura N, Shozuhara H, Onodera H, Itoyama Y, Kogure K. (1995) Interleukin-1 as a pathogenetic mediator of ischemic brain damage in rats. Stroke, 26: 676-680.
369. Bonestroo HJ, Nijboer CH, van Velthoven CT, Kavelaars A, Hack CE, van Bel F, Heijnen CJ. (2013) Cerebral and hepatic inflammatory response after neonatal hypoxia-ischemia in newborn rats. Dev Neurosci, 35: 197-211.
370. Hagberg H, Gilland E, Bona E, Hanson LA, Hahin-Zoric M, Blennow M, Holst M, McRae A, Soder O. (1996) Enhanced expression of interleukin (IL)-1 and IL-6 messenger RNA and bioactive protein after hypoxia-ischemia in neonatal rats. Pediatr Res, 40: 603-609.
371. Jin Y, Silverman AJ, Vannucci SJ. (2007) Mast cell stabilization limits hypoxic-ischemic brain damage in the immature rat. Dev Neurosci, 29: 373-384.
372. Sun Y, Calvert JW, Zhang JH. (2005) Neonatal hypoxia/ischemia is associated with decreased inflammatory mediators after erythropoietin administration.

Stroke, 36: 1672-1678.
373. Hedtjarn M, Leverin AL, Eriksson K, Blomgren K, Mallard C, Hagberg H. (2002) Interleukin-18 involvement in hypoxic-ischemic brain injury. J Neurosci, 22: 5910-5919.
374. Vela JM, Molina-Holgado E, Arevalo-Martin A, Almazan G, Guaza C. (2002) Interleukin-1 regulates proliferation and differentiation of oligodendrocyte progenitor cells. Mol Cell Neurosci, 20: 489-502.
375. Kadhim H, Tabarki B, De Prez C, Rona AM, Sebire G. (2002) Interleukin-2 in the pathogenesis of perinatal white matter damage. Neurology, 58: 1125-1128.
376. Betz AL, Schielke GP, Yang GY. (1996) Interleukin-1 in cerebral ischemia. Keio J Med, 45: 230-237.
377. Bonmann E, Suschek C, Spranger M, Kolb-Bachofen V. (1997) The dominant role of exogenous or endogenous interleukin-1 beta on expression and activity of inducible nitric oxide synthase in rat microvascular brain endothelial cells. Neurosci Lett, 230: 109-112.
378. Friedlander RM, Gagliardini V, Hara H, Fink KB, Li W, MacDonald G, Fishman MC, Greenberg AH, Moskowitz MA, Yuan J. (1997) Expression of a dominant negative mutant of interleukin-1 beta converting enzyme in transgenic mice prevents neuronal cell death induced by trophic factor withdrawal and ischemic brain injury. J Exp Med, 185: 933-940.
379. Liu F, Ooi VE, Fung MC. (1999) Analysis of immunomodulating cytokine mRNAs in the mouse induced by mushroom polysaccharides. Life Sci, 64: 1005-1011.
380. Garcia JH, Liu KF, Relton JK. (1995) Interleukin-1 receptor antagonist decreases the number of necrotic neurons in rats with middle cerebral artery occlusion. Am J Pathol, 147: 1477-1486.
381. Loddick SA, Rothwell NJ. (1996) Neuroprotective effects of human recombinant interleukin-1 receptor antagonist in focal cerebral ischaemia in the rat. J Cereb Blood Flow Metab, 16: 932-940.
382. Relton JK, Rothwell NJ. (1992) Interleukin-1 receptor antagonist inhibits ischaemic and excitotoxic neuronal damage in the rat. Brain Res Bull, 29: 243-246.
383. Merrill JE. (1992) Tumor necrosis factor alpha, interleukin 1 and related cytokines in brain development: normal and pathological. Dev Neurosci, 14: 1-10.
384. Moore JP, Kitchen SG, Pugach P, Zack JA. (2004) The CCR5 and CXCR4 coreceptors--central to understanding the transmission and pathogenesis of human immunodeficiency virus type 1 infection. AIDS Res Hum Retroviruses, 20: 111-126.
385. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. (1999) Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature, 401: 708-712.
386. Bergsbaken T, Fink SL, Cookson BT. (2009) Pyroptosis: host cell death and inflammation. Nat Rev Microbiol, 7: 99-109.
387. Ventura A, Meissner A, Dillon CP, McManus M, Sharp PA, Van Parijs L, Jaenisch R, Jacks T. (2004) Cre-lox-regulated conditional RNA interference from transgenes. Proc Natl Acad Sci U S A, 101: 10380-10385.
388. Zielinski CE, Mele F, Aschenbrenner D, Jarrossay D, Ronchi F, Gattorno M,

Monticelli S, Lanzavecchia A, Sallusto F. (2012) Pathogen-induced human TH17 cells produce IFN-γ or IL-10 and are regulated by IL-1β. Nature, 484: 514.

389. Doitsh G, Galloway NL, Geng X, Yang Z, Monroe KM, Zepeda O, Hunt PW, Hatano H, Sowinski S, Munoz-Arias I, Greene WC. (2014) Cell death by pyroptosis drives CD4 T-cell depletion in HIV-1 infection. Nature, 505: 509-514.
390. Perregaux D, Gabel CA. (1994) Interleukin-1 beta maturation and release in response to ATP and nigericin. Evidence that potassium depletion mediated by these agents is a necessary and common feature of their activity. J Biol Chem, 269: 15195-15203.
391. Arbore G, West EE, Spolski R, Robertson AAB, Klos A, Rheinheimer C, Dutow P, Woodruff TM, Yu ZX, O'Neill LA, Coll RC, Sher A, Leonard WJ, Kohl J, Monk P, Cooper MA, Arno M, Afzali B, Lachmann HJ, Cope AP, Mayer-Barber KD, Kemper C. (2016) T helper 1 immunity requires complement-driven NLRP3 inflammasome activity in CD4(+) T cells. Science, 352: aad1210.
392. Grivel JC, Margolis LB. (1999) CCR5- and CXCR4-tropic HIV-1 are equally cytopathic for their T-cell targets in human lymphoid tissue. Nat Med, 5: 344-346.
393. Martin-Ancel A, Garcia-Alix A, Pascual-Salcedo D, Cabanas F, Valcarce M, Quero J. (1997) Interleukin-6 in the cerebrospinal fluid after perinatal asphyxia is related to early and late neurological manifestations. Pediatrics, 100: 789-794.
394. Suzuki S, Tanaka K, Suzuki N. (2009) Ambivalent aspects of interleukin-6 in cerebral ischemia: inflammatory versus neurotrophic aspects. J Cereb Blood Flow Metab, 29: 464-479.
395. Szaflarski J, Burtrum D, Silverstein FS. (1995) Cerebral hypoxia-ischemia stimulates cytokine gene expression in perinatal rats. Stroke, 26: 1093-1100.
396. Ali C, Nicole O, Docagne F, Lesne S, MacKenzie ET, Nouvelot A, Buisson A, Vivien D. (2000) Ischemia-induced interleukin-6 as a potential endogenous neuroprotective cytokine against NMDA receptor-mediated excitotoxicity in the brain. J Cereb Blood Flow Metab, 20: 956-966.
397. Maeda Y, Matsumoto M, Hori O, Kuwabara K, Ogawa S, Yan SD, Ohtsuki T, Kinoshita T, Kamada T, Stern DM. (1994) Hypoxia/reoxygenation-mediated induction of astrocyte interleukin 6: a paracrine mechanism potentially enhancing neuron survival. J Exp Med, 180: 2297-2308.
398. Hama T, Kushima Y, Miyamoto M, Kubota M, Takei N, Hatanaka H. (1991) Interleukin-6 improves the survival of mesencephalic catecholaminergic and septal cholinergic neurons from postnatal, two-week-old rats in cultures. Neuroscience, 40: 445-452.
399. Hirota H, Kiyama H, Kishimoto T, Taga T. (1996) Accelerated Nerve Regeneration in Mice by upregulated expression of interleukin (IL) 6 and IL-6 receptor after trauma. J Exp Med, 183: 2627-2634.
400. Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD, McClanahan T, Kastelein RA, Cua DJ. (2005) IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. J Exp Med, 201: 233-240.

401. Srenathan U, Steel K, Taams LS. (2016) IL-17+ CD8+ T cells: Differentiation, phenotype and role in inflammatory disease. Immunol Lett, 178: 20-26.
402. Shichita T, Sugiyama Y, Ooboshi H, Sugimori H, Nakagawa R, Takada I, Iwaki T, Okada Y, Iida M, Cua DJ, Iwakura Y, Yoshimura A. (2009) Pivotal role of cerebral interleukin-17-producing gammadeltaT cells in the delayed phase of ischemic brain injury. Nat Med, 15: 946-950.
403. Yang D, Sun YY, Bhaumik SK, Li Y, Baumann JM, Lin X, Zhang Y, Lin SH, Dunn RS, Liu CY, Shie FS, Lee YH, Wills-Karp M, Chougnet CA, Kallapur SG, Lewkowich IP, Lindquist DM, Murali-Krishna K, Kuan CY. (2014) Blocking lymphocyte trafficking with FTY720 prevents inflammation-sensitized hypoxic-ischemic brain injury in newborns. J Neurosci, 34: 16467-16481.
404. Balabanov R, Strand K, Kemper A, Lee JY, Popko B. (2006) Suppressor of cytokine signaling 1 expression protects oligodendrocytes from the deleterious effects of interferon-gamma. J Neurosci, 26: 5143-5152.
405. Mann SA, Versmold B, Marx R, Stahlhofen S, Dietzel ID, Heumann R, Berger R. (2008) Corticosteroids reverse cytokine-induced block of survival and differentiation of oligodendrocyte progenitor cells from rats. J Neuroinflammation, 5: 39.
406. Mesples B, Plaisant F, Gressens P. (2003) Effects of interleukin-10 on neonatal excitotoxic brain lesions in mice. Brain Res Dev Brain Res, 141: 25-32.
407. Hansen-Pupp I, Harling S, Berg AC, Cilio C, Hellstrom-Westas L, Ley D. (2005) Circulating interferon-gamma and white matter brain damage in preterm infants. Pediatr Res, 58: 946-952.
408. Dihne M, Block F. (2001) Focal ischemia induces transient expression of IL-6 in the substantia nigra pars reticulata. Brain Res, 889: 165-173.
409. Loddick SA, Turnbull AV, Rothwell NJ. (1998) Cerebral interleukin-6 is neuroprotective during permanent focal cerebral ischemia in the rat. J Cereb Blood Flow Metab, 18: 176-179.
410. Stroemer RP, Rothwell NJ. (1998) Exacerbation of ischemic brain damage by localized striatal injection of interleukin-1beta in the rat. J Cereb Blood Flow Metab, 18: 833-839.
411. Pulera MR, Adams LM, Liu H, Santos DG, Nishimura RN, Yang F, Cole GM, Wasterlain CG. (1998) Apoptosis in a neonatal rat model of cerebral hypoxia-ischemia. Stroke, 29: 2622-2630.
412. Tarkowski E, Rosengren L, Blomstrand C, Jensen C, Ekholm S, Tarkowski A. (1999) Intrathecal expression of proteins regulating apoptosis in acute stroke. Stroke, 30: 321-327.
413. Nakazawa T, Nakazawa C, Matsubara A, Noda K, Hisatomi T, She H, Michaud N, Hafezi-Moghadam A, Miller JW, Benowitz LI. (2006) Tumor necrosis factor-alpha mediates oligodendrocyte death and delayed retinal ganglion cell loss in a mouse model of glaucoma. J Neurosci, 26: 12633-12641.
414. Arnett HA, Mason J, Marino M, Suzuki K, Matsushima GK, Ting JP. (2001) TNF alpha promotes proliferation of oligodendrocyte progenitors and remyelination. Nat Neurosci, 4: 1116-1122.
415. Cammer W, Zhang H. (1999) Maturation of oligodendrocytes is more sensitive to TNF alpha than is survival of precursors and immature oligodendrocytes. J Neuroimmunol, 97: 37-42.

416. Jurewicz A, Matysiak M, Tybor K, Kilianek L, Raine CS, Selmaj K. (2005) Tumour necrosis factor-induced death of adult human oligodendrocytes is mediated by apoptosis inducing factor. Brain, 128: 2675-2688.
417. Pang Y, Cai Z, Rhodes PG. (2005) Effect of tumor necrosis factor-alpha on developing optic nerve oligodendrocytes in culture. J Neurosci Res, 80: 226-234.
418. Deng YY, Lu J, Ling EA, Kaur C. (2010) Microglia-derived macrophage colony stimulating factor promotes generation of proinflammatory cytokines by astrocytes in the periventricular white matter in the hypoxic neonatal brain. Brain Pathol, 20: 909-925.
419. Silveira RC, Procianoy RS. (2003) Interleukin-6 and tumor necrosis factor-alpha levels in plasma and cerebrospinal fluid of term newborn infants with hypoxic-ischemic encephalopathy. J Pediatr, 143: 625-629.
420. Foster-Barber A, Dickens B, Ferriero DM. (2001) Human perinatal asphyxia: correlation of neonatal cytokines with MRI and outcome. Dev Neurosci, 23: 213-218.
421. Oygur N, Sonmez O, Saka O, Yegin O. (1998) Predictive value of plasma and cerebrospinal fluid tumour necrosis factor-alpha and interleukin-1 beta concentrations on outcome of full term infants with hypoxic-ischaemic encephalopathy. Arch Dis Child Fetal Neonatal Ed, 79: F190-193.
422. Shohami E, Bass R, Wallach D, Yamin A, Gallily R. (1996) Inhibition of tumor necrosis factor alpha (TNFalpha) activity in rat brain is associated with cerebroprotection after closed head injury. J Cereb Blood Flow Metab, 16: 378-384.
423. Dietrich WD, Busto R, Bethea JR. (1999) Postischemic hypothermia and IL-10 treatment provide long-lasting neuroprotection of CA1 hippocampus following transient global ischemia in rats. Exp Neurol, 158: 444-450.
424. Grilli M, Barbieri I, Basudev H, Brusa R, Casati C, Lozza G, Ongini E. (2000) Interleukin-10 modulates neuronal threshold of vulnerability to ischaemic damage. Eur J Neurosci, 12: 2265-2272.
425. Molina-Holgado E, Vela JM, Arevalo-Martin A, Guaza C. (2001) LPS/IFN-gamma cytotoxicity in oligodendroglial cells: role of nitric oxide and protection by the anti-inflammatory cytokine IL-10. Eur J Neurosci, 13: 493-502.
426. Froen JF, Munkeby BH, Stray-Pedersen B, Saugstad OD. (2002) Interleukin-10 reverses acute detrimental effects of endotoxin-induced inflammation on perinatal cerebral hypoxia-ischemia. Brain Res, 942: 87-94.
427. Dordelmann M, Kerk J, Dressler F, Brinkhaus MJ, Bartels DB, Dammann CE, Dork T, Dammann O. (2006) Interleukin-10 high producer allele and ultrasound-defined periventricular white matter abnormalities in preterm infants: a preliminary study. Neuropediatrics, 37: 130-136.
428. Gorelik L, Flavell RA. (2000) Abrogation of TGFbeta signaling in T cells leads to spontaneous T cell differentiation and autoimmune disease. Immunity, 12: 171-181.
429. Kehrl JH, Wakefield LM, Roberts AB, Jakowlew S, Alvarez-Mon M, Derynck R, Sporn MB, Fauci AS. (1986) Production of transforming growth factor beta by human T lymphocytes and its potential role in the regulation of T cell growth. J Exp Med, 163: 1037-1050.

430. Kulkarni AB, Huh CG, Becker D, Geiser A, Lyght M, Flanders KC, Roberts AB, Sporn MB, Ward JM, Karlsson S. (1993) Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. Proc Natl Acad Sci U S A, 90: 770-774.
431. Laouar Y, Sutterwala FS, Gorelik L, Flavell RA. (2005) Transforming growth factor-beta controls T helper type 1 cell development through regulation of natural killer cell interferon-gamma. Nat Immunol, 6: 600-607.
432. Lucas PJ, Kim SJ, Melby SJ, Gress RE. (2000) Disruption of T cell homeostasis in mice expressing a T cell-specific dominant negative transforming growth factor beta II receptor. J Exp Med, 191: 1187-1196.
433. Wan YY, Flavell RA. (2007) 'Yin-Yang' functions of transforming growth factor-beta and T regulatory cells in immune regulation. Immunol Rev, 220: 199-213.
434. Yang X, Letterio JJ, Lechleider RJ, Chen L, Hayman R, Gu H, Roberts AB, Deng C. (1999) Targeted disruption of SMAD3 results in impaired mucosal immunity and diminished T cell responsiveness to TGF-beta. EMBO J, 18: 1280-1291.
435. Li MO, Wan YY, Flavell RA. (2007) T cell-produced transforming growth factor-beta1 controls T cell tolerance and regulates Th1- and Th17-cell differentiation. Immunity, 26: 579-591.
436. Gorelik L, Flavell RA. (2002) Transforming growth factor-beta in T-cell biology. Nat Rev Immunol, 2: 46-53.
437. Sad S, Mosmann TR. (1994) Single IL-2-secreting precursor CD4 T cell can develop into either Th1 or Th2 cytokine secretion phenotype. J Immunol, 153: 3514-3522.
438. Kitani A, Fuss I, Nakamura K, Kumaki F, Usui T, Strober W. (2003) Transforming growth factor (TGF)-beta1-producing regulatory T cells induce Smad-mediated interleukin 10 secretion that facilitates coordinated immunoregulatory activity and amelioration of TGF-beta1-mediated fibrosis. J Exp Med, 198: 1179-1188.
439. Fallarino F, Grohmann U, Hwang KW, Orabona C, Vacca C, Bianchi R, Belladonna ML, Fioretti MC, Alegre ML, Puccetti P. (2003) Modulation of tryptophan catabolism by regulatory T cells. Nat Immunol, 4: 1206-1212.
440. Tadokoro CE, Shakhar G, Shen S, Ding Y, Lino AC, Maraver A, Lafaille JJ, Dustin ML. (2006) Regulatory T cells inhibit stable contacts between CD4+ T cells and dendritic cells in vivo. J Exp Med, 203: 505-511.
441. Dobolyi A, Vincze C, Pal G, Lovas G. (2012) The neuroprotective functions of transforming growth factor beta proteins. Int J Mol Sci, 13: 8219-8258.
442. Zhu Y, Culmsee C, Roth-Eichhorn S, Krieglstein J. (2001) Beta(2)-adrenoceptor stimulation enhances latent transforming growth factor-beta-binding protein-1 and transforming growth factor-beta1 expression in rat hippocampus after transient forebrain ischemia. Neuroscience, 107: 593-602.
443. Gross CE, Bednar MM, Howard DB, Sporn MB. (1993) Transforming growth factor-beta 1 reduces infarct size after experimental cerebral ischemia in a rabbit model. Stroke, 24: 558-562.
444. Henrich-Noack P, Prehn JH, Krieglstein J. (1994) Neuroprotective effects of TGF-beta 1. J Neural Transm Suppl, 43: 33-45.
445. McNeill H, Williams C, Guan J, Dragunow M, Lawlor P, Sirimanne E, Nikolics

K, Gluckman P. (1994) Neuronal rescue with transforming growth factor-beta 1 after hypoxic-ischaemic brain injury. Neuroreport, 5: 901-904.
446. Guan J, Miller OT, Waugh KM, McCarthy DC, Gluckman PD, Gunn AJ. (2004) TGF beta-1 and neurological function after hypoxia-ischemia in adult rats. Neuroreport, 15: 961-964.
447. Ma M, Ma Y, Yi X, Guo R, Zhu W, Fan X, Xu G, Frey WH, 2nd, Liu X. (2008) Intranasal delivery of transforming growth factor-beta1 in mice after stroke reduces infarct volume and increases neurogenesis in the subventricular zone. BMC Neurosci, 9: 117.
448. Ruocco A, Nicole O, Docagne F, Ali C, Chazalviel L, Komesli S, Yablonsky F, Roussel S, MacKenzie ET, Vivien D, Buisson A. (1999) A transforming growth factor-beta antagonist unmasks the neuroprotective role of this endogenous cytokine in excitotoxic and ischemic brain injury. J Cereb Blood Flow Metab, 19: 1345-1353.
449. Schwarcz R, Pellicciari R. (2002) Manipulation of brain kynurenines: glial targets, neuronal effects, and clinical opportunities. J Pharmacol Exp Ther, 303: 1-10.
450. Stone TW. (2001) Kynurenines in the CNS: from endogenous obscurity to therapeutic importance. Prog Neurobiol, 64: 185-218.
451. Marchi M, Risso F, Viola C, Cavazzani P, Raiteri M. (2002) Direct evidence that release-stimulating alpha7* nicotinic cholinergic receptors are localized on human and rat brain glutamatergic axon terminals. J Neurochem, 80: 1071-1078.
452. Lugo-Huitron R, Blanco-Ayala T, Ugalde-Muniz P, Carrillo-Mora P, Pedraza-Chaverri J, Silva-Adaya D, Maldonado PD, Torres I, Pinzon E, Ortiz-Islas E, Lopez T, Garcia E, Pineda B, Torres-Ramos M, Santamaria A, La Cruz VP. (2011) On the antioxidant properties of kynurenic acid: free radical scavenging activity and inhibition of oxidative stress. Neurotoxicol Teratol, 33: 538-547.
453. Soliman H, Mediavilla-Varela M, Antonia S. (2010) Indoleamine 2,3-dioxygenase: is it an immune suppressor? Cancer J, 16: 354-359.
454. Mandi Y, Vecsei L. (2012) The kynurenine system and immunoregulation. J Neural Transm (Vienna), 119: 197-209.
455. Vecsei L, Szalardy L, Fulop F, Toldi J. (2013) Kynurenines in the CNS: recent advances and new questions. Nat Rev Drug Discov, 12: 64-82.
456. Buck RH, Cordle CT, Thomas DJ, Winship TR, Schaller JP, Dugle JE. (2002) Longitudinal study of intracellular T cell cytokine production in infants compared to adults. Clin Exp Immunol, 128: 490-497.
457. Jason J, Archibald LK, Nwanyanwu OC, Byrd MG, Kazembe PN, Dobbie H, Jarvis WR. (2001) Comparison of serum and cell-specific cytokines in humans. Clin Diagn Lab Immunol, 8: 1097-1103.
458. Counsell SJ, Tranter SL, Rutherford MA. (2010) Magnetic resonance imaging of brain injury in the high-risk term infant. Semin Perinatol, 34: 67-78.
459. Cseko AJ, Bango M, Lakatos P, Kardasi J, Pusztai L, Szabo M. (2013) Accuracy of amplitude-integrated electroencephalography in the prediction of neurodevelopmental outcome in asphyxiated infants receiving hypothermia treatment. Acta Paediatr, 102: 707-711.
460. Bosmans JM, Neri E, Ratib O, Kahn CE, Jr. (2015) Structured reporting: a fusion reactor hungry for fuel. Insights Imaging, 6: 129-132.

461. Marcovici PA, Taylor GA. (2014) Journal Club: Structured radiology reports are more complete and more effective than unstructured reports. AJR Am J Roentgenol, 203: 1265-1271.
462. Herve C, Beyne P, Jamault H, Delacoux E. (1996) Determination of tryptophan and its kynurenine pathway metabolites in human serum by high-performance liquid chromatography with simultaneous ultraviolet and fluorimetric detection. J Chromatogr B Biomed Appl, 675: 157-161.
463. Hama T, Miyamoto M, Tsukui H, Nishio C, Hatanaka H. (1989) Interleukin-6 as a neurotrophic factor for promoting the survival of cultured basal forebrain cholinergic neurons from postnatal rats. Neurosci Lett, 104: 340-344.
464. Mehler MF, Marmur R, Gross R, Mabie PC, Zang Z, Papavasiliou A, Kessler JA. (1995) Cytokines regulate the cellular phenotype of developing neural lineage species. Int J Dev Neurosci, 13: 213-240.
465. Satoh T, Nakamura S, Taga T, Matsuda T, Hirano T, Kishimoto T, Kaziro Y. (1988) Induction of neuronal differentiation in PC12 cells by B-cell stimulatory factor 2/interleukin 6. Mol Cell Biol, 8: 3546-3549.
466. Relton JK, Martin D, Thompson RC, Russell DA. (1996) Peripheral administration of Interleukin-1 Receptor antagonist inhibits brain damage after focal cerebral ischemia in the rat. Exp Neurol, 138: 206-213.
467. Lin CY, Chang YC, Wang ST, Lee TY, Lin CF, Huang CC. (2010) Altered inflammatory responses in preterm children with cerebral palsy. Ann Neurol, 68: 204-212.
468. Rothhammer V, Heink S, Petermann F, Srivastava R, Claussen MC, Hemmer B, Korn T. (2011) Th17 lymphocytes traffic to the central nervous system independently of alpha4 integrin expression during EAE. J Exp Med, 208: 2465-2476.
469. Hu Y, Zheng Y, Wu Y, Ni B, Shi S. (2014) Imbalance between IL-17A-producing cells and regulatory T cells during ischemic stroke. Mediators Inflamm, 2014: 813045.
470. Tzartos JS, Friese MA, Craner MJ, Palace J, Newcombe J, Esiri MM, Fugger L. (2008) Interleukin-17 production in central nervous system-infiltrating T cells and glial cells is associated with active disease in multiple sclerosis. Am J Pathol, 172: 146-155.
471. Trandem K, Zhao J, Fleming E, Perlman S. (2011) Highly activated cytotoxic CD8 T cells express protective IL-10 at the peak of coronavirus-induced encephalitis. J Immunol, 186: 3642-3652.

12 PUBLICATIONS

Publications directly related to the PhD dissertation

Cumulative impact factor: 10.386

1) Bajnok A, Berta L, Orbán C, Tulassay T, Toldi G. (2018) Cytokine production pattern of T lymphocytes in neonatal arterial ischemic stroke during the first month of life-a case study. J Neuroinflammation. 15(1):191. **IF: 5.193**

2) Bajnok A, Berta L, Orbán C, Veres G, Zádori D, Barta H, Méder Ü, Vécsei L, Tulassay T, Szabó M, Toldi G. (2017) Distinct cytokine patterns may regulate the severity of neonatal asphyxia-an observational study. J Neuroinflammation. 14(1):244. **IF: 5.193**

Publications not related to the PhD dissertation:

Cumulative impact factor: 42.072, as first author: 6.615

1) Bajnok A, Ivanova M, Rigó J Jr, Toldi G. (2017) The Distribution of Activation Markers and Selectins on Peripheral T Lymphocytes in Preeclampsia. Mediators Inflamm. 2017:8045161. **IF: 3.549**

2) Bajnok A, Kaposi A, Kovács L, Vásárhelyi B, Balog A, Toldi G. (2013) Analysis by flow cytometry of calcium influx kinetics in peripheral lymphocytes of patients with rheumatoid arthritis. Cytometry A. 83(3):287-93. **IF: 3.066**

3) Orbán C, Vásárhelyi Z, Bajnok A, Sava F, Toldi G. Effects of caffeine and phosphodiesterase inhibitors on activation of neonatal T lymphocytes. Immunobiology. 223(11):627-633. **IF: 2.873**

4) Dulic S, Vasarhelyi Z, Bajnok A, Szalay B, Toldi G, Kovacs L, Balog A (2018). The Impact of Anti-TNF Therapy on CD4+ and CD8+ Cell Subsets in Ankylosing Spondylitis. Pathobiology. 85(3):201-210. **IF: 1.592**

5) Orbán C, D Szabó, A Bajnok, B Vásárhelyi, T Tulassay, A Arató, G Veres, G Toldi. (2017) Altered activation of peripheral CD8+ T cells in pediatric Crohn's disease. Immunol Lett, 185:48-51. **IF: 2.483**

6) Legány N, G Toldi, C Orbán, N Megyes, A Bajnok, A Balog. Calcium influx kinetics, and the features of potassium channels of peripheral lymphocytes in primary Sjögren's syndrome. (2106) Immunobiology 221:1266-1272. **IF:2.720**

7) Orbán C, D Szabó, A Bajnok, B Vásárhelyi, T Tulassay, A Arató, G Veres, G Toldi. (2016) Altered calcium influx of peripheral Th2 cells in pediatric Crohn's disease: infliximab may normalize activation patterns. Oncotarget. 7(29):44966-44974. **IF: 5.168**

8) Orban C, E Perez-Garcia, A Bajnok, G McBean, G Toldi, A Blanco-Fernandez. Real time kinetic flow cytometry measurements of cellular parameter changes evoked by nanosecond pulsed electric field. (2016) Cytometry Part A 89:472-479. **IF: 3.222**

9) Folyovich A, Biczó D, Bajnok A, Bessenyei D, Kis I, Gimesi-Országh J, Béres-Molnár AK, Toldi G. (2016) Higher Incidence of Stroke on the Last Day of the Month in Hungary-a Role for Psychosocial Factors and Financial Insecurity? J Stroke Cerebrovasc Dis. 25(5):1192-1195. **IF: 1.517**

10) Toldi G, ZE Vásárhelyi, J Rigó Jr, C Orbán, Z Tamássy, A Bajnok, T Shima, S Saito, A Molvarec. Prevalence of Regulatory T-Cell Subtypes in Preeclampsia. (2015) American Journal of Reproductive Immunology 74:110-115. **IF: 2.916**

11) Grozdics E, Berta L, Bajnok A, Veres G, Ilisz I, Klivényi P, Rigó J Jr, Vécsei L, Tulassay T, Toldi G. (2014) B7 costimulation and intracellular indoleamine-2,3-dioxygenase (IDO) expression in peripheral blood of healthy pregnant and non-pregnant women. BMC Pregnancy Childbirth. 14:306. **IF: 2.190**

12) Orbán C, A Bajnok, B Vasarhelyi, T Tulassay, G Toldi. (2014) Different calcium influx characteristics upon Kv1.3 and IKCa1 potassium channel inhibition in T helper subsets. Cytometry Part A, 85: 636-641. **IF: 2.928**

13) Folyovich A, Biro E, Orban C, Bajnok A, Varga V, Beres-Molnar AK, Vasarhelyi B, Toldi G. Relevance of novel inflammatory markers in stroke-induced immunosuppression. (2014) BMC Neurology 14:41. **IF:2.040**

14) Folyovich A, Biró E, Orbán C, Bajnok A, Vásárhelyi B, Toldi G. Kv1.3 lymphocyte potassium channel inhibition as a potential novel therapeutic target in acute ischemic stroke. (2014) CNS & Neurological Disorders-Drug Targets 13:801-806. **IF: 2.628**

15) Orbán C, E Biro, E Grozdics, A Bajnok, G Toldi. (2013) Modulation of T lymphocyte calcium influx patterns via the inhibition of Kv1.3 and IKCa1 potassium channels in autoimmune disorders. Frontiers in Immunology 4: 3.

16) Toldi G, Bajnok A, Dobi D, Kaposi A, Kovács L, Vásárhelyi B, Balog A. (2013) The effects of Kv1.3 and IKCa1 potassium channel inhibition on calcium influx of human peripheral T lymphocytes in rheumatoid arthritis. Immunobiology. 218(3):311-6. **IF: 3.180**

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