Biomarkers in cerebrospinal fluid of neonates at risk of brain injury

Association with death and disability

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Thesis for the degree of Philosophiae Doctor

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SCHOOL OF HEALTH SCIENCES
FACULTY OF MEDICINE

Lífefni í mænuvökva nýbura: forspárgildi fyrir heilaskaða

Tengsl við lifun og röskun á taugaþroska

Kristín Leifsdóttir

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var yfir 20% árið 1850
er nú kominn niður í 0,4%

það eru þessi 0,4%

Hanna Óladóttir

Þessi bók er tileinkuð drengjunum mínum Kristjáni Júlíani og Jóhanni Hauki,
-sem fylla líf mitt af sólskini.

Ágrip

Bakgrunnur

Börn sem verða fyrir súrefnisskorti í fæðingu og börn sem fæðast fyrir fulla meðgöngu eiga á hættu að lifa ekki af eða hljóta varanlegan heilaskaða. Hættan á þessum fylgikvillum hefur ekki minnkað á síðustu áratugum þrátt fyrir miklar læknisfræðilegar framfarir. Súrefnisskortur í fæðingu og önnur áföll setja í gang efnaferla í heilanum sem leiða til truflunar á starfsemi taugafruma og jafnvel taugafrumudauða. Sýkingar eru algengur fylgikvilli þess að fæðast fyrir tímann og eru ein helsta orsök hárrar dánartíðni fyrirbura á nýburaskeiði. Þær tengjast einnig truflunum í öndun og blóðrás vegna áhrifa bólguefna á stjórnstöð í heilastofni. Bólguferli gegna lykilhlutverki sem orsakavaldur heilaskaða bæði eftir sýkingar og við súrefnisþurrð. Mikilvægt er að hafa í huga að óþroskaður heili nýbura, einkum fyrirbura, er viðkvæmur fyrir alls kyns áreiti á nýburaskeiði. Truflun á þroska heilans, jafnvel án sjáanlegs meins í heilavef, getur valdið því að barnið hljóti varanlegar fatlanir. Mögulegt er að mæla í lífsýnum efni svo sem bólguefni og prótein sem tengjast hinum flóknu efnaferlum heilaskaðans og/eða heilaþroskans.

Markmið og tilgátur

Markmið þessa verkefnis var að finna leiðir til að greina heilaskaða og meta áhættu á taugaþroskaröskun hjá nýburum. Til að ná þeim markmiðum vildum við finna lífefni í mænuvökva sem mundu geta: i) gefið upplýsingar um útbreiðslu og alvarleika heilaskaða hjá fullburða börnum sem fengið höfðu súrefnisskort í fæðingu sem og horfur þessara barna, ii) spáð fyrir um framtíðarhorfur hjá fyrirburum, og iii) útskýrt truflanir á hjartslætti og öndun hjá nýburum með sýkingar.

Megintilgátur okkar voru í fyrsta lagi, að magn bólguefna i) mundi mælast hækkað í mænuvökva hjá börnum sem lent höfðu í súrefnisskorti í fæðingu og tengdust bæði meiri alvarleika skaðans og verri horfum, ii) að þau væru hærri hjá fyrirburum í samanburði við fullburða börn sem ekki höfðu hlotið súrefnisskort í fæðingu, og að iii) hækkun á bólguefninu Prostaglandin E_2 (PGE_2) mundi tengjast, ekki einungis súrefnisskorti í fæðingu, heldur einnig truflunum í öndun og hjartslætti hjá sýktum nýburum.

Í öðru lagi að önnur lífefni, sem sérstaklega tengjast hinum ýmsu efnaferlum heilaskaða hjá nýburum, væru hækkuð í mænuvökva hjá þeim

börnum sem orðið höfðu fyrir súrefnisskorti í fæðingu og að þau tengdust alvarleika skaðans og horfum barnanna.

Í þriðja lagi að próteinmynstur í mænuvökva væri mismunandi hjá fyrirburum annars vegar og fullburða börnum hins vegar og að magn próteina sem tengjast hinum ýmsu efnaferlum í heilaþroskanum hefðu forspárgildi um taugaþroskaraskanir hjá fyrirburum.

Aðferðir

Til að kanna tilgátur okkar mældum við lífefni í mænuvökva fullburða barna sem orðið höfðu fyrir súrefnisskorti í fæðingu, hjá fyrirburum með eða án sýkingar og hjá fullburða börnum í viðmiðunarhópi.

Fullburða börn sem voru innlögð á nýburagjörgæsludeild Karolinska sjúkrahússins í Stockholm á tímabilinu 2000 til 2004 vegna fósturköfnunar voru höfð í rannsóknum I, III og IV. Öll börnin uppfylltu skilyrði fyrir heilakvilla af völdum súrefnisskorts í fæðingu og var mænuástunga gerð sem liður í almennri uppvinnslu. Viðmiðunarhópur var börn, einnig fædd eftir fulla meðgöngu, sem voru inniliggjandi á Karolinska sjúkrahúsinu vegna gruns um sýkingu en höfðu ekki lent í súrefnisskorti í fæðingu og voru ekki með heilakvilla. Fyrirburar innlagðir á Karolinska sjúkrahúsið á árunum 2002 til 2004 sem voru mænustungnir vegna gruns um sýkingu voru í rannsóknum II og V. Fullburða börn sem lágu á sama tíma á Karolinska sjúkrahúsinu og voru einnig mænustungin vegna gruns um sýkingu voru viðmiðunarhópur.

Mænuvökvi frá þessum börnum var rannsakaður með ELISA aðferð. Magn PGE_2 og PGE_2 myndefnis (PGEM) var mælt í rannsókn I og II og magn interleukin 6 (IL-6) og fas-ligand (FasL) í rannsókn III. Prótein-svipgerð í mænuvökva í rannsóknum IV og V var mæld með tækni sem mælir mörg prótein samtímis og byggir á sækni próteina í fyrirfram gerða mótefnalausn. Mótefnalausnin var gerð úr próteinögnum sem valdar voru úr próteingagnabanka. Magn 178 próteina var mælt í sýnunum.

Öllum börnum var fylgt eftir til 18-24 mánaða aldurs.

Niðurstöður

Helstu niðurstöður okkar voru, í fyrsta lagi, að magn bólguefna eykst í mænuvökva, bæði við blóðsýkingar og fósturköfnun, og hafa notagildi við greiningu á sjúkdómsástandi og forspárgildi um horfur sjúklinga. Í öðru lagi, að ýmis prótein sem tengjast efnaferlum í meingerð heilaskaða af völdum súrefnisskorts safnast upp í mænuvökva eftir fósturköfnun. Aukið magn þessara próteina tengist verri heilaskaða og magn þeirra í mænuvökva hafði tengsl við framtíðarhorfur barnanna. Í þriðja lagi sýndum við að prótein-svipgerð í mænuvökva er ekki eins hjá fyrirburum og hjá fullburða börnum.

Einnig sáum við að hjá fyrirburum sem greindust með taugaþroskaraskanir fyrir tveggja ára aldur var skortur á ýmsum próteinum sem taka þátt í þroska heilans.

Í rannsóknum I og II mældust bólguefnið PGE_2 og afleiða þess PGEM hækkuð í mænuvökva. Hjá börnum með sýkingar (rannsókn II) höfðu þessi lífefni notagildi sem greiningartæki snemma í sjúkdómsferlinu og tengdust truflun á hjartslætti og öndunarstarfsemi. Hjá börnum með heilakvilla eftir fósturköfnun (rannsókn I) voru tengsl á milli magns þessara lífefna og alvarleika heilaskaðans, bæði gráðu heilakvilla og horfur barnanna. IL-6 er annað bólguefni sem mældist í auknu magni í mænuvökva þeirra barna sem urðu fyrir súrefnisskort í fæðingu og tengdist það verri heilaskaða (rannsókn III). Í þeirri rannsókn voru einnig tengsl á milli magns FasL og alvarleika heilaskaða en það er lífefni sem tekur þátt í dauða taugafruma í flókinni meingerð heilaskaða eftir súrefnisskort. IL-6 og FasL notuð saman spáðu fyrir um alvarleika heilaskaðans, gráðu heilakvilla og horfur, með miklu næmi og sértæki. Próteinmynstur í mænuvökva barna með heilakvilla eftir fósturköfnun (rannsókn IV) einkenndist af bólguferlum og öðrum próteinum sem tengjast efnaferlum heilaskaða af völdum súrefnisskorts. Hækkað magn þessara próteina tengdist meiri einkennum um heilakvilla og verri langtímahorfum þessara barna. Próteinmynstur í mænuvökva fyrirbura var öðruvísi en hjá fullburða börnum (rannsókn V), og var þar sérstaklega áberandi hækkun á bólgupróteinum hjá fyrirburum. Í þeirri rannsókn var magn próteina sem tengdust heilaþroskanum minna í mænuvökva þeirra fyrirbura sem greindust með óeðlilegan taugaþroska við 18-24 mánaða leiðréttan aldur en hjá fyrirburum sem voru með eðlilegan taugaþroska.

Ályktun

Útfrá þessum niðurstöðum ályktum við að ýmis bólguefni og önnur lífefni í mænuvökva geta verið gagnleg við mat á ástandi og horfum nýbura sem eru í hættu á heilaskaða eða taugaþroskaröskun. Þau geta verið gagnleg i) við greiningu á heilakvilla af völdum súrefniskorts í fæðingu, ii) við greiningar á sýkingum ii) við mat á alvarleika heilaskaða hjá nýburum sem hlotið hafa fósturköfnun og við mat á þörfinni fyrir meðferð og iii) við mat á framtíðarhorfum fyrirbura, og fullburða barna með heilakvilla.

Að auki gefa þessar niðurstöður mikilvægar upplýsingar um meingerð heilaskaðans, sem gætu nýst við þróun á nýjum meðferðarúrræðum.

Lykilorð:

Fósturköfnun, blóðsýking, heilakvilli orsakaður af súrefnisþurrð, lífefni, mænuvökvi.

Abstract

Background

Prematurity with its complications and hypoxic-ischemic encephalopathy (HIE) following birth asphyxia in full-term infants are the most important contributors to a high incidence of early neonatal mortality and lifelong neurodevelopmental disabilities originating in the neonatal period. Devastating long-term outcomes have not decreased markedly within the last decades despite extensive medical advances. Various injurious processes are stimulated by external insult and can lead to brain cell damage. This includes inflammatory processes, which are important contributors to brain injury in both sepsis and hypoxia-ischemia. Sepsis is a common complication of prematurity and the most frequent cause of mortality in the neonatal period. It is associated with cardiorespiratory disturbances, as a result of a disrupted autonomic control in the brain stem. It is important to bear in mind the vulnerability of the developing brain, especially of the preterm infant. Diverse postnatal events may alter normal brain development and lead to an adverse neurodevelopmental outcome, even without apparent lesion in the brain tissue. Measuring biomarkers in body fluids that correlate with the processes of brain injury and/or brain development is feasible.

Aims

The focus of this thesis was to diagnose perinatal brain injury and identify infants at risk of adverse neurodevelopmental outcomes. Specifically, the aim was to discover biomarkers in cerebrospinal fluid (CSF) that would: i) identify the degree of HIE and the clinical outcome following perinatal asphyxia, ii) predict the neurodevelopmental outcome of preterm infants, and iii) correlate with the onset of neonatal sepsis and cardiorespiratory disturbances.

We first hypothesized that neuroinflammation would be observed following birth asphyxia and that inflammatory mediators could serve as biomarkers of the degree of HIE and the clinical outcome of the patients. Furthermore, the inflammatory mediator prostaglandin E_2 (PGE_2) would correlate with the consequences of perinatal asphyxia and early neonatal sepsis and autonomic dysfunction. Secondly, the levels of brain-specific proteins, including apoptotic markers, cell structural proteins, and synaptic proteins, in CSF, could serve as diagnostic and prognostic biomarkers in HIE. Thirdly, the proteome in preterm infants would differ from that in term-born infants and a specific protein signature would predict adverse neurodevelopmental outcomes in the preterm infants.

Methods

We analyzed CSF of term infants with HIE, infected and noninfected preterm infants, and term control infants to test these hypotheses. Term infants who were cared for at the neonatal intensive care unit at the Karolinska Hospital in Stockholm due to perinatal asphyxia, between 2000 and 2004, were enrolled prospectively into studies I, III, and IV. Included infants all fulfilled the criteria of perinatal asphyxia and had a lumbar puncture performed on clinical indications. Term infants without asphyxia or any other pathology in the brain who were cared for at the Karolinska Hospital at the same time for suspected infection served as controls. Preterm infants cared for at the neonatal intensive care unit at Karolinska Hospital between 2002 and 2004 and who underwent clinically indicated lumbar puncture were prospectively enrolled into studies II and V. Term infants with suspected infection served as controls.

CSF was analyzed from patients and controls. ELISA technique was utilized to measure PGE_2 and prostaglandin E_2 metabolite (PGEM) in studies I and III and for measuring interleukin 6 (IL-6) and fas-ligand (FasL) in study III. In studies IV and V, proteomic profiling was performed utilizing an antibody affinity-based proteomic array. We measured the relative abundances of 178 unique proteins. The antibodies for the suspension bead array were produced from protein fragments chosen from a protein database for the analysis.

Neurological follow-up was performed at 18-24 months in all infants.

Results

Our main findings were, firstly, that inflammatory biomarkers were increased in CSF in response to both asphyxia and sepsis and had diagnostic as well as prognostic value. Secondly, unique brain-specific proteins were increased in CSF of asphyxiated infants and correlated with the extent of brain injury and clinical outcome. Thirdly, decreased levels of neurodevelopmentally related proteins correlated with unfavorable neurodevelopmental outcomes of preterm infants.

More specifically, the inflammatory mediators PGE_2 and its more stable metabolite PGEM were found in increased amounts in studies I and II. They had diagnostic value in sepsis and correlated with cardiorespiratory disturbances (study II). In infants who had suffered perinatal asphyxia, they correlated with the HIE degree and the outcome of patients (study I). IL-6 is another inflammatory mediator, that was found in an increased amount in asphyxiated infants in study III and correlated with HIE degree and clinical outcome. In that study, FasL, which is an apoptotic marker and associated

with brain cell death in hypoxic-ischemic (HI) brain injury, was found to increase as well and predicted the extent of brain injury and clinical outcome. IL-6 and FasL together predicted HIE degree and outcome with high sensitivity and specificity. Protein profiles in study IV revealed an upregulation of inflammatory pathways as well as other pathways involved in HI brain injury. Several proteins predicted the HIE degree and the clinical outcome of the infants. In study V preterm infants had a different protein profile than term-born infants. Furthermore, depressed levels of several neurodevelopmentally related proteins correlated with adverse outcomes in the preterm group.

Conclusion

From these results, it may be concluded that several inflammatory- as well as structural and functional brain-specific biomarkers could serve as diagnostic tools in infants at risk of brain injury. Specifically, they could; i) have diagnostic value in HIE and sepsis, ii) help identify infants with HIE grade II and III, who would benefit from neuroprotective treatment after perinatal asphyxia, and iii) have value in predicting the risk of adverse neurological outcomes in infants with HIE and preterm infants.

Furthermore, they reflect the injurious processes in HIE and expand the knowledge of the neurodevelopmental processes in the developing brain. This might be important for the invention of future neuroprotective methods.

Keywords:

Asphyxia, sepsis, hypoxic-ischemic encephalopathy (HIE), biomarkers, cerebrospinal fluid (CSF).

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Contents

List of abbreviations

AA ..... Arachidonic Acid
ABD ..... Apnea, Bradycardia and Desaturation
ADHD ..... Attention deficit hyperactivity disorder
AIF ..... Apoptosis Inducing Factor
ALDOC ..... Aldolase C
AMER ..... APC Membrane Recruitment Protein
AMPA α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionicAcid
APC ..... Antigen Presenting Cell
AQP4 ..... Aquaporin-4
ARPP21 ..... cAMP regulated Phosphoprotein 21
ASD ..... Autsm Spectrum Disorder
ATP6V1G2 ..... Adenosine Triphosphatease V-type subunit G2
AUC ..... Area Under the Curve
BAX ..... Bcl-2-Associated X protein
BBB ..... Blood Brain Barrier
Bcl2 ..... B cell lymphoma 2
BDNF ..... Brain Derived Neurotrophic Factor
BE ..... Base Excess
BGT ..... Basal Ganglia andThalamus
BSID ..... Bayley Scales of Infant and Toddler Development
C11orf87 ..... Chromosomes 11 open reading frame 87
C5 ..... Complement 5
C9 ..... Complement 9
CAMs ..... Cell Adhesion Molecules
Caskin-1 ..... Calcium-dependent Serine interacting protein Kinase-1
CFB ..... Complement factor B
CNS ..... Central Nervous System
COX ..... Cyclooxygenase
CP ..... Cerebral Palsy
CRP ..... C-Reactive Protein
CSF ..... Cerebropinal fluid
DAMPs ..... Danger-associated molecular pathways
dMRI ..... diffusion Magnetic ResonanceI maging
DSCAM ..... Down Syndrome Cell Adhesion Molecule
EAA ..... Excitatory Amino Acid
EIA ..... Enzyme Immunoassay
ELISA ..... Enzyme Linked Immunosorbent Assay

EPO ..... .Erythropoietin
EPRs..... E-Prostanoid Receptors
ER..... Endoplasmic Reticulum
FADD ..... Fas-Associated Death Domain
Fas..... .Fas receptor
FasL..... Fas-ligand
GA..... Gestational Age
GABA..... Gamma Aminobutyric Acid
GFAP ..... Glial Fibrillary Acidic Protein
GLP-1 ..... Glucagon-Like Peptide-1
HI ..... Hypoxic Ischemia
HIE..... Hypoxic Ischemic Encephalopathy
HPA ..... Human Protein Atlas
ICAM-1..... Intercellular Adhesion Molecule-1
IQR ..... Interquartile Range
IGF..... Insulin-like Growth Factor
IL-1..... Interleukin 1 beta
IL-6..... Interleukin 6
IL-6R ..... Interleukin 6 receptor
IVH..... Intraventricular Haemorrhage
LDH..... Lactate Dehydrogenase
LP ..... Lumbar Puncture
LPS ..... Lipopolysaccarides
MASP2..... Mannan-binding lectin Serine Protease 2
MBP ..... Myelin Basic Protein
MDI ..... Mental Developmental Index
MFI..... Median Fluorescent Intensities
MgSO4..... Magnesium Sulfate
MMPs..... Matrix Metalloproteinases
mPGES-1..... microsomal Prostaglandin E Synthase-1
MRI ..... Magnetic Resonance Imaging
mRNA ..... messenger RNA
miRNAs..... microRNAs
MRS..... Magnetic Resonance Spectroscopy
NCAN..... Neurocan core protein
NE..... Neonatal Encephalopahty
NICU ..... Neonatal Intensive Care Unit
NMDA ..... N-Methyl-D-Aspartate
NMDAR..... N-Methyl-D-Aspartate Receptor
NNT ..... Number Needed to Treat
NPTX1 ..... Neuronal Pentraxin-1
NSE ..... Neuron Specific Enolase

OL ..... Oligodendrocytes
OPCs ..... Oligodendrocyte Progenitor Cells
PARP ..... Poly (ADP-ribose) polymerase-1
PCA ..... Principal Component Analysis
PET ..... Positron Emission Tomography
PGE2 ..... Prostaglandin E2
PGEM ..... Prostaglandin E2 Metabolite
PGs ..... Prostaglandines
PHVD ..... Post Haemorragic Ventricular Dilatation
preOL ..... premyelinating Oligodendrocytes
PrESTs ..... Protein Fragments
RCT ..... Randomised Controlled Trial
rIL-1RA ..... recombinant Interleukin-1 Receptor Antagonost
ROC ..... Receiver-Operator Characteristic
ROP ..... Retinopathy of Prematurity
ROS ..... Reactive Oxygen Species
RTN1 ..... Reticulon-1
SEM ..... Standard Error of the Mean
Sez6 ..... Seizure protein 6
SLC12A5 ..... Solute Carrier Family 12 member 5
SPTAN ..... Alpha-II Spectrin
SV2A ..... Synaptic Vesicle Glycoprotein 2A
SVZ ..... Subventricular Zone
TBI ..... Traumatic Brain Injury
TBR1 ..... neuron-specific T-box transcription factor
TGF-β ..... Transformin Growth Factor-beta
TH ..... Therapeutic Hypothermia
TLR ..... Toll Like Receptor
TNF-α ..... Tumor Necrosis Factor-alpha
tPA ..... tissue Plasminogen Activator
VCAM ..... Vascular Cell Adhesion Molecule
VZ ..... Ventricular Zone
WHO ..... World Health Organization
WMD ..... White Matter Damage
WMI ..... White Matter Injury

List of figures

List of tables

List of original papers

This thesis is based on the following papers which are referred to in the text by their Roman numerals (I-V):

I. Björk L, Leifsdottir K, Saha S, Herlenius E. **PGE_2 - metabolite levels in CSF correlate to HIE score and outcome after perinatal asphyxia**. Acta Paediatr. 2013 Nov;102(11):1041-1047

II. Siljehav V, Hofstetter AM, Leifsdottir K, Herlenius E. **Prostaglandin E_2 Mediates Cardiorespiratory Disturbances during Infection in Neonates**. J Pediatr. 2015 Dec;167(6):1207-1213

III. Leifsdottir K, Mehmet H, Eksborg S, Herlenius E. **Fas-ligand and interleukin-6 in the cerebrospinal fluid are early predictors of hypoxic-ischemic encephalopathy and long-term outcomes after birth asphyxia in term infants**. J Neuroinflammation. 2018 Aug 8;15(1):223

IV. Leifsdottir K, Thelin E, Lassarén P, Siljehav V, Nilsson P, Eksborg S, Herlenius E. **Proteomic profiles in cerebrospinal fluid predicted death and disability in term infants with perinatal asphyxia: A pilot study.** Acta Paediatrica, 2022 Feb 2;

V. Leifsdottir K, Jost K, Siljehav V, Thelin E, Lassarén P, Nilsson P, Eksborg S, Herlenius E. **The cerebrospinal fluid proteome of preterm infants predicts future neurodevelopmental outcomes.** Submitted.

Declaration of contribution

Paper I

Leifsdottir K, Bjork L and Herlenius E were responsible for the study design. Leifsdottir K was responsible for recruiting the patients and collecting and processing the CSF samples. Björk L and Saha S did the ELISA analysis of the samples. Leifsdottir K was responsible for collecting and analysing the clinical data. Leifsdottir K, Björk L and Herlenius E were responsible for analysing the data and writing the paper.

Paper II

Leifsdottir K participated in patient recruitment, CSF collection and storage. Siljehav V and Hofstetter AM did the analysis of the samples and were responsible for analysing the data. Leifsdottir K, Siljehav V and Herlenius E were responsible for writing and reviewing the manuscript.

Paper III

Leifsdottir K and Mehmet H were responsible for designing and planning the research. Leifsdottir K was responsible for the patient recruitment and collection of the CSF samples as well as processing and storing them. Leifdottir K did the ELISA analysis of the samples. Leifsdottir K, Mehmet H, Eksborg S and Herlenius E analyzed the data. Eksborg S was responsible for the statistics. Leifsdottir K, Memet H and Herlenius E were responsible for writing the paper.

Paper IV

Leifsdottir K, Thelin E, Siljehav V and Herlenius E were responsible for designing and planning the research. Leifsdottir K did the patient recruitment, collection of CSF, processing and storing the CSF and collection of clinical data. Nilson P and Thelin E were responsible for the array analysis of the samples. Leifsdottir K, Eksborg S, Lassarén P and Thelin E analyzed the data. Leifsdottir K, Thelin E, Siljehav V and Herlenius E were responsible for writing the paper.

Paper V

Leifsdottir K, Siljehav V and Herlenius E recruited the patients and collected the CSF samples. They also did the processing and storing of the CSF. Siljehav V and Jost K, Leifsdottir K and Herlenius E participated in collecting clinical data. Thelin E and Nilson P were responsible for the array analysis of the samples. Leifsdottir K, Eksborg S, Lassarén P and Herlenius E analyzed the data. Eksborg S was responsible for the statistics. Leifsdottir K, Siljehav V, Jost K, Herlenius E, Haraldsson Á, and Thelin E were responsible for writing and reviewing the manuscript.

1 Introduction

Preterm birth and birth-related insults in term infants contribute to a high incidence of early neonatal mortality, and the risk of lifelong neurodevelopmental disabilities is high for those who survive (Bell et al., 2022; Cheong et al., 2021). The outcome has not improved noticably in the last years despite advances in the obstetrical and neonatal care (Johnson & Marlow, 2017). The brain of the fetus and newborn is developing at a very rapid rate. This makes newborn infants, especially the preterm infant, vulnerable to a diversity of external stimuli around birth and in the neonatal period. The burgeoning corpus of information on neurodevelopmental impairment in preterm infants and term-born infants who suffered from birth complications requires an awareness.

Infants born prematurely

Around 15 million infants are born preterm every year, with the rate ranging from about 5% to 18% in different parts of the world, and rates are increasing (Blencowe et al., 2012). The incidence of mortality and morbidity is highest in the lowest gestational ages. Moderate or late preterm, from 32 to 37 completed gestational weeks, is the biggest group and has the best prognosis of preterm infants (Kerstjens, de Winter, Bocca-Tjeertes, Bos, & Reijneveld, 2012). Very preterm infants, from 28 to 32 gestational weeks, are 1-2%, and extremely preterm infants, born after less than 28 completed gestational weeks, are 0,4-0,7% of all live births. With improved maternal and neonatal care, the survival rates of the extremely preterm infants have increased in higher-income countries over the past decades (Norman et al., 2019; B. J. Stoll et al., 2015). Nevertheless, the mortality rate is still high in this group. It is very varying between countries (Helenius et al., 2017). Neurodevelopmental impairments occur in many surviving infants (Bell et al., 2022; Glass et al., 2015). Most outcome studies show that between five and 10% of preterm survivors who are born after less than 32 gestational weeks have major motor impairments, with highest risk in the lowest gestational ages (Spittle, Cameron, Doyle, Cheong, & Victorian Infant Collaborative Study, 2018). Cognitive disabilities are seen at much higher rates in this group, as high as 25-50% in very preterm infants, and the rates are even higher in the lowest gestational ages (Amer et al., 2018).

Invasive infections in neonates

Neonatal sepsis, which is a common complication of preterm birth, is associated with a high risk of mortality in neonates (Oza, Lawn, Hogan, Mathers, & Cousens, 2015). It may affect the neurological outcome of surviving infants (Pek, Gan, et al., 2020; Schlapbach et al., 2011). Results of a meta-analysis of the effect of neonatal sepsis on the developing brain are pending (Pek, Yap, et al., 2020). Infection, even distant from the brain can through inflammatory mediators induce neuroinflammation which may result in altered neurodevelopment, especially in the immature brain (Allred et al., 2017). It may also disrupt brainstem respiratory control, leading to apneas which is a common clinical symptom in neonatal infection, especially in preterm infants. Furthermore, exposure to infectious or inflammatory stimuli can make the brain vulnerable, sensitizing the brain to a secondary insult and thereby exacerbating the injurious cascades that lead to disturbed brain function (Fleiss et al., 2015). Inflammation is one of the key molecular pathways of the HI brain injury (Dammann & O'Shea, 2008).

Hypoxic-Ischemic Encephalopathy (HIE)

After premature birth intrapartum complications with oxygen deprivation, leading to hypoxic-ischemic (HI) brain injury, is the second most common contributor to early neonatal mortality (Perin et al., 2022). Between 1 and 3 infants per 1000 live birth is diagnosed with HIE each year in high-income countries (Azzopardi et al., 2014). After introduction of therapeutic hypothermia (TH), a reduced death or severe disability by 15% was confirmed in large randomized controlled trials (RCTs) (Edwards et al., 2010; Jacobs et al., 2013). Of those who survived the initial injury, an additional 25% developed lifelong serious disabilities, including cerebral palsy (CP), epilepsy, sensory- and cognitive deficits. More recent RCT data indicate a further reduction in risk of adverse outcome from 45% to 29%, mainly related to a reduction in mortality, from 25% to 10% (Shankaran et al., 2017). In low-income countries, the rate of HIE is as high as 26 per 1000 infants and with fewer survivors (Kurinczuk, White-Koning, & Badawi, 2010).

The emphasis of this thesis

The assessment of infants at risk of brain injury is currently based on clinical evaluation, electrophysiology, and brain imaging. Methodologies that provide insight into the pathophysiological processes of brain injury are rapidly progressing. The biomarker science expectantly will change the care of neonates at risk of serious complications (Graham, Everett, Delpech, & Northington, 2018). Our prime focus was to define biochemical markers in

cerebrospinal fluid (CSF) that could be used in concert with the currently available diagnostic tools and that would improve the predictability of the assessment of infants at risk of brain injury. Also, to shed light on the pathology of brain injury and dysmaturation. This is a topic of major importance as it may aid in the attempt to improve survival and long-term outcome for the many suffering infants.

1.1 Brain development and vulnerability

Neurodevelopment is an evolving process from early in intrauterine life and throughout postnatal life. At the 20th week of gestation, the brain weighs only 10% of the term brain and the structure is very immature. Maturational vulnerability of the rapidly expanding cortex in preterm infants has been established (Matthews et al., 2018). From the 20th gestational week, and especially during the third trimester, impressive developmental changes occur on molecular, neurochemical, and structural levels (Lefevre et al., 2016). These are processes like neurogenesis, neuronal migration, brain network formation, gliogenesis, angiogenesis, and synaptogenesis. During early fetal development, neurogenesis and migration are the most prominent of these processes, but subsequently or at around 27 weeks of gestation synaptogenesis becomes very active and it persists into the second year of life (Johnston et al., 2009).

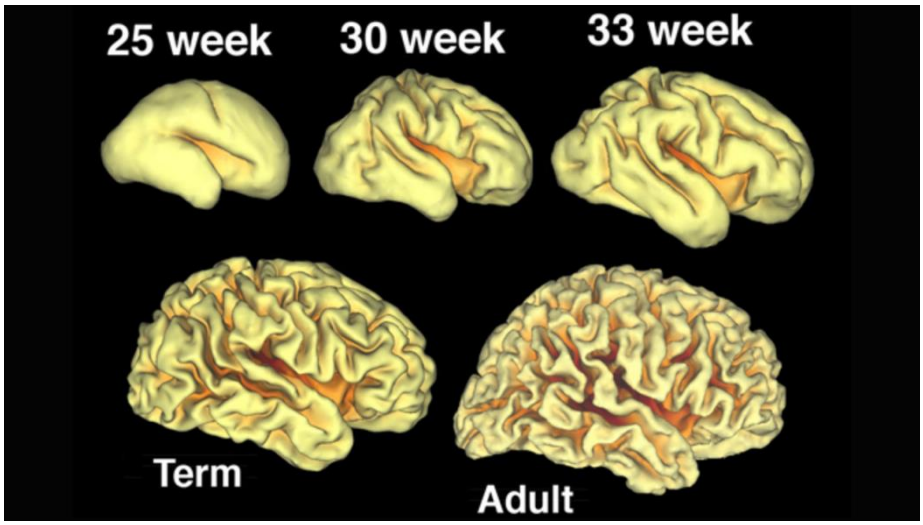


Figure 1. Cortical folding patterns in preterm and term infants.
Reprinted with permission from David Van Essen, Jason Hill, Terrie Inder, and Jeff Neil.

1.1.1 Neurons

Axons and subplate neurons

Neurons are generated from around the 16th week of gestation. Rapidly dividing neuronal progenitor cells migrate from germinative zones into the subplate area, which is a neuronal layer VI of the cortex and a waiting area, before they differentiate to reach the cortex (Hoerder-Suabedissen & Molnar, 2015). The subplate neurons reach maximal development at 27th to 30th weeks of gestation, at the time when axons reach the deep layer of the cortex. This coincides with the extremely vulnerable gestational ages in preterm infants (Bystron, Blakemore, & Rakic, 2008). They then proliferate and migrate to the upper layers of the cortex, continuing to the end of the gestation (Kostovic & Judas, 2006).

Synaptogenesis, formation of cortex and thalamus

Communication between neurons is essential for brain development. Synapses appear from 24th to the 32nd week of gestation (Kostovic & Jovanov-Milosevic, 2006). A mature synaptic network is formed when glutamatergic neurons in the subplate receive excitatory input from the thalamus and make excitatory synapsis with neurons higher up in the neocortex (Friauf & Shatz, 1991). This maturational process which includes excitatory neurotransmitters is highly important for the formation of the cortex and of the thalamus and other deep nuclei (T. Inder et al., 2005).

Late migrating GABAergic neurons and microcircuitry

A second phase of neuronal generation has been documented, which likely continues well into the third trimester (Inta et al., 2008). These neurons are mainly inhibitory (GABAergic) (Xu et al., 2011). In the cortex, the later arriving GABAergic interneurons constitute 20-30% of all cortical neurons. Micro-circuitry between inhibitory GABAergic neurons and glutamatergic excitatory neurons is highly important for the functional development of the cerebral cortex (Garcia-Marin, Blazquez-Llorca, Rodriguez, Gonzalez-Soriano, & DeFelipe, 2010).

1.1.2 Neuroglia

Pre-Oligodendrocytes (preOL)

Disturbed white matter development may result from dysregulation of glial cells (Back & Rosenberg, 2014). Oligodendrocytes (OLs) produce myelin around neuronal axons for their protection and for optimizing their function, during the last part of gestation and into the adult life (H.C. & D.L., 2002).

Before term age, the OLs are predominantly in a pre-myelinating phase. Pre-oligodendrocytes (PreOLs) have a maturation-dependent vulnerability to oxidative stress, infections, and other insults (Back, 2014). After brain insult, an aberrant repair system regenerates the cells, which leads to a disruptive developmental program with failure in differentiation and myelin production. This aberrant process is associated with diffuse white matter injury (WMI), which is a common brain pathology in preterm infants (Back, 2017). WMI is also is found in term infants with brain hypoxic brain injury (S. P. Miller & Ferriero, 2009). As neurons migrate into the cortex through white matter at this vulnerable stage they risk being injured at the same time.

Microglia

Microglia are the so-called “brain macrophages”, with an important role in the brains’ immune defense, having either pro-inflammatory or anti-inflammatory functions. They are also important in normal brain development. Microglia are at a peak abundance in the third trimester of gestation. They enhance synaptogenesis and neural circuit formation (Hammond, Robinton, & Stevens, 2018). Microglia stimulate pre-OL proliferation, but they can also induce OL death (Volpe, 2009).

Astrocytes

Astrocytes are also part of the brain defense and neuro repair. They clear excessive glutamate and trigger anti-inflammatory cytokine and growth factor production (Sofroniew & Vinters, 2010). They are also important in the crosstalk between brain cells, synaptogenesis, and for the energy turnover of neurons (Mamczur et al., 2015). In response to brain insult, a robust gliogenesis occurs which may have both beneficial or detrimental effects on neuronal survival and remodeling of the brain.

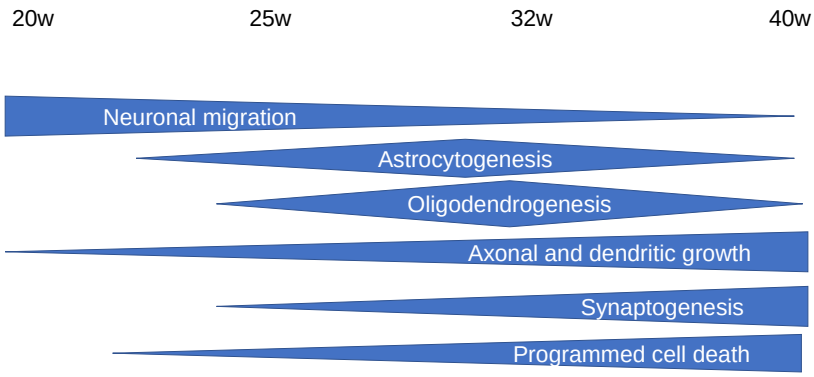


Figure 2. The evolving processes of neurodevelopment throughout gestation.

1.1.3 Plasticity

Neuroplasticity is the capacity of the brain to change or remodel itself in response to a stimulus or an insult. The word “plasticity” comes from the Greek word “plastos” which means molded. Developmental neuroplasticity is the ability of developmental processes to variate. Intrinsic mechanisms regulate the developmental processes to maintain normal brain development. Nevertheless, the developing brain is highly vulnerable to external events (Ismail, Fatemi, & Johnston, 2017). Neuroplasticity generally has a trophic role, and the purpose of reactive plasticity is to “overcome” an insult. Nevertheless, the plasticity may render the brain more vulnerable to injury and worsen the outcome (Johnston, Nakajima, & Hagberg, 2002). Indeed, although the developing brains‘ capacity to change is more than the adults‘ brain, it often has worse neurodevelopmental outcomes following brain injury. This is dependent on the vulnerability of the plasticity in various developmental processes at the time of the insult (Giza & Prins, 2006).

Insult to the developing brain may have an impact on multiple neurodevelopmental processes including neurogenesis, neuronal maturation, and establishment of neural networks as well as glial maturation and myelination. Therefore, the neurological outcome is ultimately defined to a large extent by the timing of the insult to the developing brain and the endogenous responses (Kinney, 2009; S. J. Vannucci & Hagberg, 2004).

1.1.4 Brain dysmaturation

Subplate neurons are susceptible to the effects of hypoxic-ischemic events, as has been observed in neonatal hypoxia animal models (McQuillen, Sheldon, Shatz, & Ferriero, 2003). Alterations in the subplate during critical periods alter the activity-dependent thalamocortical afferent development, which in turn has potentially adverse effects on both cortical and thalamic development (Kostovic, Judas, & Sedmak, 2011). It has been shown that interference with the establishment of brain connections between different structural sites causes disrupted brain growth. This has been suggested by volumetric magnetic resonance imaging (MRI) (Y. Zhang et al., 2015), and correlated with adverse neurodevelopmental outcome (Counsell & Boardman, 2005; Thompson et al., 2019). Nevertheless, reduced brain volumes cannot fully explain the effect of prematurity on cognitive and neurobehavioral difficulties. It is suggested that alterations in microstructure, which are not captured on MRI-defined volume measures, may be as important (Gozdas et al., 2018). This is considered secondary to white matter damage (WMD) in the developing brain, but is even more important in determining the outcomes (Volpe, 2019a).

1.1.5 Mediators of neurodevelopment

Various cerebral tissue enriched proteins are essential for brain development. Evidence suggests that altered expression of developmental genes are associated with altered neurodevelopment in preterm infants (Evans, Karama, Vasung, & Iturria-Medina, 2017). The alteration in gene expression may result from external stimuli like infection (Hagberg et al., 2015). Kuban et al showed that higher neurotrophin protein levels correlated with larger brain volumes and better cognitive outcomes in preterm infants (Kuban et al., 2018). In the same patient group upregulation of neuroinflammatory proteins was correlated with worse outcomes and lower brain volumes (Kuban et al., 2019). Protein levels change dramatically during the first weeks of life and the change is determined by the type of postnatal stimuli that the infant is exposed to (Zhong et al., 2021). There is growing evidence of the importance of various proteins in normal brain development and neuroplasticity. Notably, there is also evidence of the involvement of inflammatory proteins in the normal brain development (Biggins, Brennan, Taylor, Woodruff, & Ruitenberg, 2017; Magdalon et al., 2020). The lack of several proteins have been implicated from preclinical studies to contribute to neurological impairments like autism spectrum disorder (Daimon et al., 2015; Yook et al.,

2019), cognitive decline in the elderly (Harris et al., 2020), and psychiatric diseases (Vanes, Murray, & Nosarti, 2021).

In our proteomic study in preterm infants (*study V),* we discovered a correlation between lower protein levels in CSF and adverse neurodevelopmental outcomes. Several of these proteins are known to promote neurogenesis, neuronal migration, and plasticity.

1.2 Etiology of perinatal brain injury

It is important to notice that various factors can modify the vulnerability of the developing brain to an insult and thus affect the outcome. These include exposure to chronic infections, the nutritional status of the infant, and the developmental stage. Alterations in brain plasticity may occur like described in the previous chapter. Here below the brain insults are discussed.

1.2.1 Hypoxia-Ischemia

Neonatal encephalopathy (NE) is a clinical presentation of brain dysfunction in a term infant and does not include assumptions about the etiology (Hankins & Speer, 2003). HIE in term infants can be considered a subset of NE in cases where there is evidence of perinatal events causing asphyxia. HIE is widely used as terminology for all cases of NE. The pathophysiology of HIE will be discussed in more detail in chapter 1.3.

Encephalopathy of prematurity is not a clinical description, unlike encephalopathy in full-term infants. It applies to the pathophysiology of the injury and the diagnosis relies on neuroimaging studies (Volpe, 2019a). Multiple evidence from research within developmental neuroscience indicates a primary role of ischemia in preterm brain injury, with triggering of excitotoxic and inflammatory processes. The outcome is to a large extent determined by the brain maturity at the time of the insult (S. J. Vannucci & Hagberg, 2004; Volpe, 2019a). The incidence of HI brain injury in preterm infants varies in different studies from 4 to 48 per 1000 newborn preterm infants (Graham, Ruis, Hartman, Northington, & Fox, 2008). Nevertheless, the contribution of asphyxia in pathogenesis is debated (Gilles, Gressens, Dammann, & Leviton, 2018). In a recent study, the Apgar score was prognostic for neonatal survival in the preterm population (Cnattingius, Johansson, & Razaz, 2020). In another study acidosis in cord blood, which is another widely used marker of asphyxia, did not correlate with adverse neurologic outcome (Zaigham, Kallen, Marsal, & Olofsson, 2020). This is in contrast to HIE in term infants, where a relationship has been observed between acidosis and the risk of death or cerebral palsy (CP) (Kelly et al., 2018).

Many questions are unresolved about the origin of perinatal brain injury. It is likely that in many instances several damaging events cause NE and

encephalopathy of prematurity, rather than a single exposure (Barnett et al., 2018). Therefore, simplistic associations between single exposures and outcomes must be made with caution.

1.2.2 Neuroinflammation

The interaction between the peripheral immune system and the central nervous system (CNS) is highly important in the pathogenesis of perinatal brain injury. Inflammation can be caused by an infection, or it can be sterile. Despite different etiologies, it may contribute to brain injury in various ways. The infant may be exposed to multiple inflammatory perinatal triggers, including antenatal infections like chorioamnionitis, hypoxia-ischemia, and postnatal sepsis.

It has been suggested from preclinical studies that the effect that an infection may have in the brain is pathogen dependent. Gram-negative sepsis gives rise to more inflammation and more apoptosis in the brain than gram-positive sepsis (Falck et al., 2018). Systemic inflammation, i.e., outside the developing brain, may also contribute to brain damage in neonates by sensitizing the brain (Volpe, 2019c).

Furthermore, neuroinflammation may be triggered by danger-associated molecular patterns (DAMPs) from injured brain tissue after an insult, without an infection (Hagberg et al., 2015).

Sensitization

A model has been developed that is increasingly used to study the complex and multifactorial processes of perinatal brain injury that is called the multiple-hit model. This model involves sensitization, whereby factors that do not induce significant brain damage alone may do so in the presence of another exposure, or they make the brain more vulnerable to injurious factors (Back & Miller, 2014). Chronic fetal hypoxia, mild maternal stress, and systemic infection can all have sensitizing effects (Rousset et al., 2008). In a newborn piglet model, E-coli lipopolysaccharide infusion prior to asphyxia, potentiated the risk of hypoxic encephalopathy and adverse outcome (K. A. Martinello et al., 2019).

Epigenetic changes and miRNAs

Epigenetic changes are changes in gene activity and expression, that can be heritable but are without changes in the nucleotide sequence (Chuang & Jones, 2007). If the control processes of gene expression occur improperly it can affect brain function and development. Changes in epigenetic

programming can be caused by persisting neuroinflammation and by the influence of hypoxia in the developing brain (Liverman et al., 2006; Ma & Zhang, 2015). As a result, the vulnerable neuroplasticity may be altered, influencing the potential for repair and regeneration in the brain and altering the neurodevelopment (Y. Wu et al., 2020).

MicroRNAs (miRNAs) are small RNA molecules that regulate post-transcriptional gene expression, each miRNA having multiple target mRNAs (Bartel, 2004). They can be measured in biofluids and have been proposed as biomarkers of a variety of diseases (Reid, Kirschner, & van Zandwijk, 2011). The miRNA miR-374a has been suggested to be a biomarker of HIE as downregulation of its levels was observed in umbilical cord blood in perinatal asphyxia (Looney, Walsh, et al., 2015).

Interestingly, there is evidence of varying individual genetic susceptibility to brain injury. Especially a specific genetic regulation of inflammatory biomarkers (Harding, 2007). This calls for personalized and targeted treatment intervention and prevention.

Clinical correlations

Brain insult triggers immediate expression of inflammatory mediators, which may expand for a long time, up to many months (B. Li, Concepcion, Meng, & Zhang, 2017). This has beeen called the tertiary phase of the HI brain injury, that may result in epigenetic changes and altered neurodevelopment (Fleiss & Gressens, 2012). Furthermore, immediate rise of inflammatory markers has been observed in severe HI brain injury and is predictive of MRI abnormalities (Massaro et al., 2018; McGowan et al., 2021). In a piglet model of neonatal encephalopathy, co-existing infection exacerbates the brain injury (K. A. Martinello et al., 2019).

Preterm infants have a high incidence of exposure to inflammation during fetal life, thus very early in the brain development. Preterm birth and the risk of the adverse neurodevelopmental outcome later in life is strongly associated with chorioamnionitis and inflammation in the fetus (Edwards & Tan, 2006; Fleiss, Rivkees, & Gressens, 2019). Acute loss of pre-OLs has been associated with neuroinflammatory response to hypoxia-ischemia in preterm fetal sheep (van den Heuij et al., 2019). Inflammatory biomarkers correlate with WMD, cognitive impairments, and reduced brain volumes in preterm infants (Kuban et al., 2019). Neuroinflammation in preterm infants has also been associated with neurological and neuropsychiatric diseases throughout childhood and adulthood (Tomlinson et al., 2020; Venkatesh et al., 2020).

Blood brain barrier (BBB)

Disruption of the BBB contributes highly to the pathogenesis of brain injury. This applies both in acute hypoxia-ischemia and in the chronic neuroinflammation (Lee, Michael-Titus, & Shah, 2017). Circulating cytokines alter the permeability of BBB. Consequently, peripheral immune cells enter into the CNS, undermining the brain's immune privilege (Mallard, Ek, & Vexler, 2018). The impact of peripheral cytokines can also be mediated by cyclooxygenase (COX) isozymes located on BBB endothelial cells, which may trigger a cytotoxic neuroinflammatory reaction (Sankowski, Mader, & Valdes-Ferrer, 2015). Thus, an infection or an inflammatory process even when distant from the brain, like placental infections, vasculitis, or thrombosis, can contribute to brain injury (O'Shea et al., 2014; Volpe, 2019c).

Glial cells

Under pathological conditions, microglia can exert either deleterious or beneficial effects. Microglia are the brain's macrophages and upon phagocytosis, they produce various cytokines, including anti-inflammatory cytokines that suppress neuroinflammation and protect viable neurons. The neuroprotective effects of microglia have been confirmed in studies on the HIE mice model (S. Tsuji et al., 2020).

However, microglia may also participate in promoting neuroinflammation that leads to neuronal damage (Bhalala, Koehler, & Kannan, 2014; Volpe, 2019b). They release potentially harmful molecules like pro-inflammatory cytokines, reactive oxygen species (ROS), and matrix metalloproteinases (MMPs) (R. C. Vannucci et al., 1999). Activated microglia can even inhibit neurogenesis in the developing brain and they have been linked to white matter and axonal damage in preterm infants (Smith, Hagberg, Naylor, & Mallard, 2014).

Astrocytes are activated alongside microglia by proinflammatory mediators from injured neurons and glial cells and are a part of the host defense system (Tuttolomondo, Di Raimondo, di Sciacca, Pinto, & Licata, 2008). On the other hand, activated astrocytes can also release proinflammatory cytokines and directly induce the apoptosis of neuronal cells, thereby exacerbating an ischemic brain injury (G. Stoll, Jander, & Schroeter, 1998).

Implications

Neuroinflammation contributes to the injurious processes in concert with other pathways, or it may affect the brain development directly through

changes in epigenetics, as discussed above (Hagberg et al., 2015). It, therefore, seems plausible that modulation of the immune response would attenuate or even prevent brain damage. With that in mind, understanding the mechanism of the immune response and its role in the pathophysiology of brain damage is highly important. In all papers of this thesis increased levels of inflammatory proteins were found in CSF. In our proteomic study on asphyxiated infants (*paper IV)* the importance of immune-related proteins was confirmed by pathway analysis of CSF.

1.3 Pathophysiology of HIE

Understanding the interactive and complex biochemistry of perinatal brain injury is important. It is the foundation of improved patient care and the identification of therapeutic approaches. Hypoxia-ischemia leads to the wrecking of metabolic brain cell machinery. This is through cascades especially vulnerable in the immature brain. When triggered it can lead to energy failure, increased neuronal excitement, oxidative stress, and eventually neuronal death (Hagberg, Mallard, Rousset, & Thornton, 2014).

1.3.1 Energy failure

The hypoxic-ischemic brain damage is triggered by an insult but continues to develop over time (McLean & Ferriero, 2004). The pathophysiological processes occur in different phases, depending on energy state of the brain cells (Lorek et al., 1994). Primary energy failure starts at the time of the insult, or a few minutes after, and lasts for a few hours. This phase is characterized by a reduction in oxygen substrates, cerebral blood flow, and high-energy phosphorylated compounds. Here, excitotoxicity and free radical generation are triggered (Ferrari, Nesic, & Perez-Polo, 2010). The primary phase is followed by a latent phase, where reoxygenation and reperfusion occur. In this phase, the acute metabolic alterations in cells are completely or partly reversed and brain cells may show a recovery (Azzopardi et al., 1989; Cady, Iwata, Bainbridge, Wyatt, & Robertson, 2008). Here intervention with therapeutic hypothermia is started, in an attempt to interrupt the cascade that leads to the next phase, the secondary phase. In this phase secondary energy failure takes place. It may last for days and involves neuroinflammation, oxidative stress, excitotoxicity that leads to seizures, and permeabilization of the mitochondria. This eventually leads to brain cell death (T. E. Inder & Volpe, 2000).

The critical window of therapeutic intervention for HIE is defined within 6 hours from birth. Notably, there is considerable preclinical evidence of the benefit of starting as early as possible, as during the latent phase there is a progressive failure of the cerebral oxidative metabolism (Bennet et al., 2007; Ophelders et al., 2020). It may also in some cases be difficult to determine with precision the timing of the insult. In some infants, it may have started long before birth, sometimes with repeated or prolonged exposures. Furthermore, the intensity of the insult affects the duration of the latent phase and hence the secondary phase may start earlier following more intense insults (Wassink, Gunn, Drury, Bennet, & Gunn, 2014). In preterm infants, the identification of the timing and severity of primary and secondary insults remains even more challenging (Back & Miller, 2014).

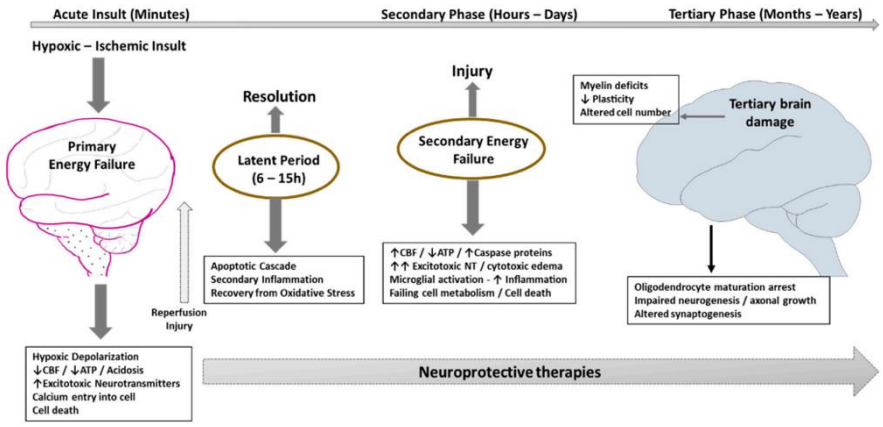


Figure 3. The different phases of HI brain injury. Ref. Nair J, Kumar VHS. Children (Basel). 2018;5(7)

After the secondary phase, there is a phase of regeneration and cell proliferation, which is essential for the ongoing neurodevelopment like myelination, synaptogenesis, and rewiring (Fleiss & Gressens, 2012). An insult to the developing brain may alter or prevent this process (Dammann & Leviton, 2007). There is considerable evidence that this tertiary, or chronic, phase of brain injury, may last for a long time (Hagberg et al., 2015).
Altered neuroplasticity and developmental processes, because of persistent inflammation and epigenetic changes, are associated with this phase (Herrera-Marschitz et al., 2014). Studies are suggesting a possibility for neuroprotective intervention during this phase to improve the post-lesion plasticity (Johnston, 2009; Tetorou, Sisa, Iqbal, Dhillon, & Hristova, 2021).

1.3.2 Molecular mechanisms

The molecular mechanisms of HI brain damage are not completely understood. Excitotoxicity and oxidative injury culminating in nerve cell death are among the major pathways as discussed here below (Millar, Shi, Hoerder-Suabedissen, & Molnar, 2017). The balance between these cascades in the processes leading to brain damage remains debated (Thornton et al., 2012). Neuroinflammation is one of the major pathways in hypoxia-ischemia, but it may also be an independent cause of perinatal brain injury and altered neurodevelopment. It was discussed separately in chapter 1.2.

Excitotoxicity

After the initial insult and during the re-oxygenation period a metabolic imbalance occurs. This is associated with cellular energy exhaustion and a rise in lactate preceding the secondary phase of energy depletion. Functional impairment of ATP-dependent membrane ionic pumps occurs, which increases the concentration of Na^+, $Ca_2^{+,}$ and Cl^- within brain cells. The neuronal resting membrane potential alters, leading to depolarization with excessive release of the amino acids (EAAs) glutamate and aspartate (Johnston, 2005). These are the excitatory neurotransmitters, and if in excess amounts in the brain the EAAs exert toxic effects on neurons and oligodendrocyte precursor cells by allowing more Ca_2+ influx into cells and triggering nitric oxide synthesis (Follett et al., 2004).

In the developing brain, the expression of EAA receptors is upregulated. They have an important role in neurodevelopment, especially neuronal synaptic plasticity (Johnston et al., 2009). This makes the immature brain extremely vulnerable to the effects of the excitotoxicity (Giza & Prins, 2006). Moreover, the selective vulnerability of different brain regions to hypoxia-ischemia, like the somatosensory cortex, thalamus, and basal ganglia, is likely to be caused by the upregulated activity of excitatory synapses of the neuronal circuits (Johnston et al., 2002).

Oxidative stress

Free radicals or reactive oxygen species (ROS) are highly reactive molecules that can damage the structure of lipids and proteins (Halliwell, 1992). The brain contains many lipids and its' antioxidant defense mechanisms are immature when under development (S. L. Miller, Wallace, & Walker, 2012). ROS accumulate when damaged cells reoxygenate (Ferriero, 2001). The mitochondrial respiration is gradually diminished during HI injury until the outer membrane becomes permeable. This leads to apoptotic cell death (Hagberg et al., 2014).

1.3.3 Nerve cell death

Apoptosis

Neuronal apoptosis is the predominant phenotype of brain cell death in the neonatal HI brain injury (Edwards et al., 1997). It is a programmed cell death primarily in the secondary phase of HI brain injury, referred to as the delayed nerve cell death (Thornton et al., 2017). Cascade of events resulting from the initial energy failure ultimately leads to apoptotic cell death through either of two pathways: intrinsic or extrinsic apoptotic pathway. Both are important in the pathogenesis of perinatal brain injury. The intrinsic pathway includes the accumulation of Ca^{2+} within the mitochondrial matrix and is characterized by increased permeability of the mitochondria and eventually DNA damage (Galluzzi, Blomgren, & Kroemer, 2009). It has been suggested in several studies that the extrinsic apoptotic pathway is important in the delayed nerve cell death.

Fas-ligand (FasL)

FasL is a natural ligand to a transmembrane death receptor, Fas (CD95/Apo-1), is implicated in this process (Broughton, Reutens, & Sobey, 2009). It is expressed in neurons and astrocytes. Upon FasL binding to Fas receptor (Fas), an intracellular death receptor domain is triggered that leads to apoptosis through the extrinsic apoptotic pathway, eventually triggering caspase 3 (H. Li, Zhu, Xu, & Yuan, 1998). In animal models, hypoxia upregulates Fas expression in the brain (van Landeghem et al., 2002). In *paper III* of this thesis death and neurodevelopmental impairment in infants with HIE were associated with increased levels of FasL in CSF (Leifsdottir, Mehmet, Eksborg, & Herlenius, 2018).

Necrosis

Necrosis is another major form of HI cell death. It has been associated with overwhelming insult and severe primary energy failure when the energy reserves of the cells are dramatically depleted. The lack of energy leads to membrane pump failure, which results in cell swelling and eventually bursting of the cell (Vanden Berghe, Linkermann, Jouan-Lanhouet, Walczak, & Vandenabeele, 2014).

Autophagy

Autophagy is another programmed cell death like apoptosis and there is a continuum between the two. The autophagic process can sequester damaged organelles to protect them from further harm, and therefore is initiated when the brain injury is repairable (Descloux, Ginet, Clarke, Puyal, & Truttmann, 2015).

1.4 Biochemical biomarkers of perinatal brain injury

Identification of infants at risk of serious complications of brain insult is challenging as the early clinical symptoms are often of limited precision. Biochemical alterations in the pathological processes of brain injury are reflected in body fluids, where they can be detected.

Biochemical biomarkers are substances that can be measured in body fluids and have an impact on or predict the diagnosis or severity of a disease or prognosis of the outcome (Safety, 2001). Throughout the past few decades, several biochemical biomarkers for early diagnosis and management of various diseases have been identified through advanced research within the genomics, proteomics, and molecular biology (Ng & Lam, 2012). They are important diagnostic tools for the identification of perinatal brain injury and the outcome prediction (Graham et al., 2018; Massaro et al., 2018). It is important to notice the time dependence of the biomarkers as the time of the insult may be difficult to determine with precision in both term and preterm brain injury. Also, therapeutic hypothermia (TH) treatment which is applied for neuroprotection in HIE alters pathological processes which makes the timing of biomarker testing even more challenging (Chavez-Valdez et al., 2021). No one metabolite gives the best prediction. Some may identify acute effects of HI brain injury and others the more prolonged and chronic effects (Blaise et al., 2017). Evidence is accumulating supporting the use of serial biomarkers or protein profiling in blood or CSF for the assessment of perinatal brain injury (Denihan et al., 2019; McGowan et al., 2021).

Proteins associated with the impaired oxidative metabolism of brain cells, cell structural proteins, apoptotic proteins, synaptic regulating proteins, proteins related to excitotoxicity, and inflammatory proteins, can all serve as biomarkers of brain injury. Also, neurotrophic biomarkers may give valuable information on brain development or disturbance thereof, and alterations of brain plasticity. In premature infants events that evolve over a prolonged period contribute highly to adverse neurodevelopmental outcomes. Therefore, there may be a long time window for interventions.

Biomarkers are important in the search for new therapeutic approaches. They can also provide information on the efficacy and safety of intervention strategies.

1.4.1 The glymphatic system

The glymphatic system removes soluble proteins and other macroscopic metabolic waste products from CNS. It is only recently discovered (Jessen, Munk, Lundgaard, & Nedergaard, 2015). The transport is facilitated by

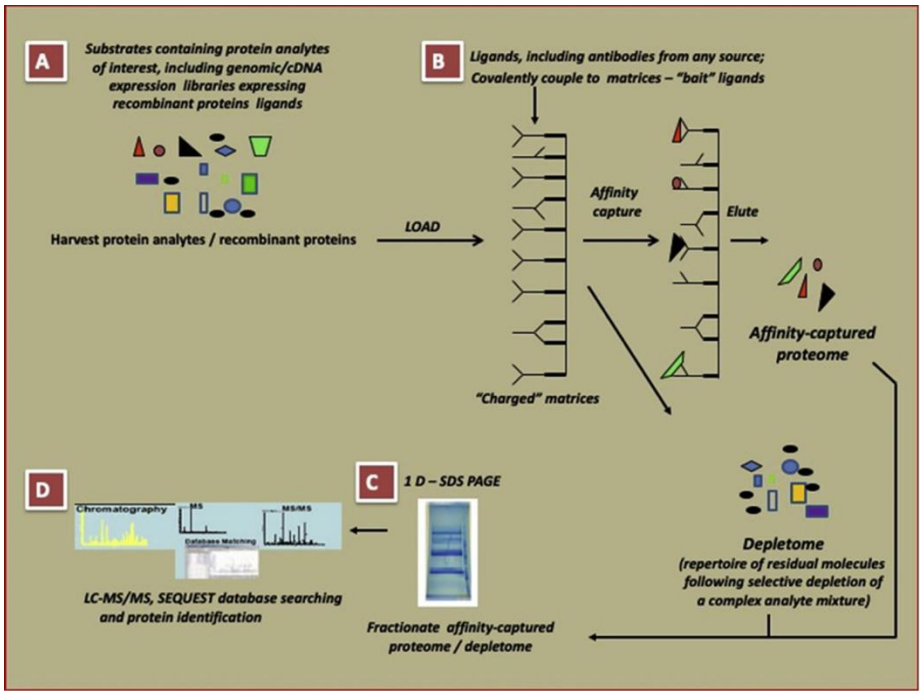
Aquaporin-4 protein (AQP4). In *study V* of the present thesis, AQP4 was one of few proteins that were observed in higher concentrations in term infants in comparison to preterm infants. This might indicate that the glymphatic system is not fully mature in preterm infants, but that remains to be investigated. In the aging brain, the loss of perivascular AQP4 polarization and dysregulation of water transport has been associated with failure in the glymphatic function and correlated with cognitive decline (Kress et al., 2014).

1.4.2 Proteomics

The overall protein content of a cell is defined as the cell's "proteome". Proteomics detect alterations in protein expression patterns in response to different signals and therefore can be utilized for the identification of various candidate diagnostic biomarkers. Proteomic-based approaches like enzyme-linked immunosorbent assay (ELISA) that are built on traditional methods have been around for decades. These assays are widely used in clinical diagnostics and are still preferred in many protein studies. ELISA offers results of a single compound per assay. It is a particularly sensitive immunoassay but not as specific, due to possible cross-reactivity between proteins in the samples. This technique was used in the first three studies of this thesis, analyzing PGE2, PGEM, IL-6, IL-6R, FasL, and Fas in the CSF samples. There have been improvements in ELISA technologies in the last few years with the production of digital device that can allow measures of a very small amount of proteins (Espana et al., 2017).

In the recent years the focus in perinatal brain injury research has shifted from analyzing one or few proteins in a sample towards profiling the protein combination (Denihan, Boylan, & Murray, 2015). The latest proteomic technologies, allow simultaneous detection of many proteins or multiplex assays, in small sample sizes. The most common technologies for multiplex assays of proteins are mass spectrometry-based methods and microarrays (Joos, TO, 2000). Antibody-based microarrays allow the targeted analysis of many proteins, in small sample sizes. This technique was utilized in *studies IV and V* of this thesis. Antibody arrays are indeed adaptations of the ELISA sandwich-style technique. A suspension bed is made which encompasses immobilized prechosen antibodies covalently bound to activated magnetic microspheres. In a complex sample, the protein abundance is measured by reading the fluorescent intensity in the samples using a flow cytometry-based laser (Luminex Corp) (Schwenk, Gry, Rimini, Uhlen, & Nilsson, 2008). The Human Protein Atlas (HPA) project (Science for Life Laboratory, Stockholm, Sweden) (Collaborators, 2021, November 18) is a valuable resource of antibody reagents for proteomics and was utilized in papers *IV and V*. It includes over 50.000 protein array validated antibodies corresponding to 17.000 unique protein-coding genes (Uhlen et al., 2010).

Figure 4. Generation of affinity captured proteome. Printed with permission from



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In perinatal brain injury, there are at present no biomarkers available for clinical use, although several have been investigated throughout the last decades. Some of the most promising ones and some observed in our studies are displayed in Table 1. Inflammatory biomarkers are described separately in the following section.

Table 1. Biomarkers of brain damage

Biomarker	Expression /source	Biological function	Contribution to the pathology	Associations
Markers *of axonal and cellular damage*				
Glial fibrillary acidic protein (GFAP)	Astrocytes	Skeletal protein in astrocytes	Marker of glial damage/death	Neonatal HIE *(Chalak et al., 2014; Massaro et al., 2018; McGowan et al., 2021).* PHVD in preterm infants *(Whitelaw, Rosengren, & Blennow, 2001). Not an early marker in HIE (Looney, Ahearne, Boylan, & Murray, 2015).*
Myelin basic protein (MBP)*	Myelin sheath	Maintenance of myelin structure and function	Marker of white matter damage	Pediatric TBI *(Beers, Berger, & Adelson, 2007).* WMI in HI animal models *(Y. Chen et al., 2013).* Neonatal HIE *(Garcia-Alix et al., 1994).*
Alpha II-spectrin (SPTAN)*	Axons	Maintains integrity of brain cells	Marker of axonal injury	Infantile epileptic encephalopathy *(Tohyama et al., 2015).* Neurodegenerative diseases *(Beijer et al., 2019).* Paediatric HI brain injury *(Jain et al., 2014).*
Osteopontin / Phosphoprotein 1 (SPP1)*	Large motor neurons and activated microglia	Extracellular matrix, T-ly activation	Inflammation, tissue repair	Cortical experimental lesion (Sugiyama et al., 2019). Early ischemic brain injury in rats (Baliga, Merrill, Shinohara, & Denhardt, 2011). Biomarker in experimental HIE (Y. Li et al., 2017)
t-Tau	Neurons	Axonal maintenance and axonal transport	Reflects neuro-axonal damage	Detecting Alzheimer´s (Jiao et al., 2021). Marker of intrapartum asphyxia (Toorell, Zetterberg, Blennow, Savman, & Hagberg, 2018). Outcome prediction after HIE (Massaro et al., 2018; McGowan et al., 2021).
Brain vascular and Blood Brain Barrier related biomarkers				
Aquaporin-4 (AQP4)*	Astrocytes and ependymal cells	Water homeostasis, synaptic plasticity	Brain oedema Neuro-inflammation	Animal models on memory loss *(Hubbard, Szu, & Binder, 2018)*

Metabolites				
Lactate dehydrogenase (LDH)	Neurons	Oxydation of lactate to pyruvate	Marker of energy failure	Neonatal HIE *(Thoresen et al., 2012)*. Outcome correlation after HIE (Chiang et al., 2016). Gray matter lesions in HIE (Yum, Moon, Youn, & Sung, 2017)
Aldolase C (ALDOC)*	Astrocytes	Cell signalling, maintaining brain energy homeostasis	Marker of astrocyte injury	Stoke and TBI *(Ottens et al., 2010).* Experimental TBI *(Thelin et al., 2018)*
ATPase H+ transporting V1 subunit G2 (ATP6V1G2)*	Neurons	Cell signalling and neurotransmitter release	Metabolic abnormalities and apoptosis	Neurodegenerative diseases *(W. X. Li et al., 2020).* Animal model on chronic progressive TBI *(Harper et al., 2020)*
Neuron speific enolase (NSE)*	Neurons and Astrocytes	Enzyme of the glycolytic pathway	Marker of neuronal and glial damage/death	Neonatal HIE *(Roka et al., 2012).* Pediatric TBI *(Berger, Beers, Richichi, Wiesman, & Adelson, 2007).*
Biomarkers with pro-apoptotic properties				
Fas-ligand (FasL)*	Immune cells and microglia	Apoptosis in normal brain development	Apoptosis in brain pathology	PHVD *(Schmitz et al., 2011)* HIE *(Leifsdottir, K., 2018)*
Reticulon-1 (RTN1)*	Neurons	Modulates voltage gated calcium channels in neurons	Excitotoxicity and apoptosis	Stroke therapy *(Gong et al., 2017).*
Synaptic proteins				
Rabphilin-3A (RPH3A)*	Neurons	Regulation of neurotransmitter release and receptor stability	Synaptic dysfunction / plasticity	Neurodegenerative diseases *(Tan et al., 2014).* Animal TBI models *(M. Chen et al., 2018)*
CASK-interacting protein 1 (Caskin1)*	Neurons	Synaptic formation and function	Synaptic dysfunction and plasticity	Experimental studies on pain signal, anxiety and memory *(Katano et al., 2018; Matsumura et al., 2010)*
Synucleins (SNCs)	Neurons and astrocytes	Regulation of neuro-transmitter release	Synaptic dysfunction and plasticity	Neurodegenerative diseases *(Sargent et al., 2018)*
Synaptic vesicle glycoprotein 2A (SV2A)*	Neurons	Regulation GABA release, seizure control.	Protection against seizure activity	Epilepsy in animal models *(Serajee & Huq, 2015)*

Neurodevelopmental proteins				
Insulin-like growth factor (IGF)	Neuroendocrine brain cells	Neurotrophic factor	Neuro-protective and trophic effects.	HI brain injury in rat model *(Brywe et al., 2005).* WMD protection in vitro *(Wood et al., 2007).*
Activin-A*	Neurons	Neurotrophic factor	Neuroprotection	HI animal HI models *(Mukerji et al., 2007).* Neonatal HIE *(Florio et al., 2004).* Umbilical marker of birth asphyxia (Fiala, Baumert, Surmiak, Walencka, & Sodowska, 2016)
Vascular endothelial factor (VEGF)*	Microglia and Astrocytes	Neurprotection Angiogenesis Neurogenesis	Inhibits apoptosis, promotes repair	*Animal HI models: Indicative of early HI brain injury, but delayed VEGF treatment enhances recovery (Dzietko, Derugin, Wendland, Vexler, & Ferriero, 2013; J. Li et al., 2020). Clinical: Anti-VEGF in ROP* (VanderVeen et al., 2017)
Seizure protein 6 (Sez6)*	Neurons	Regulates dendritic spine structure	Essential for brain development	Lack of Sez6 associated with motor impairment, memory and cognitive loss in animal models (Nash et al., 2020)
Neurocan core protein (NCAN)*		Neuro-migration	Cortical development	Lack of NCAN associated with cognitive decline and diminished brain volumes in elderly (Harris et al., 2020)

*Biomarkers that were found in CSF of preterm or term infants in the studies of this thesis in altered amounts, compared to controls.

1.4.3 Inflammatory proteins

Inflammatory proteins are a part of the host defense system. Under pathological conditions, they can have pro-inflammatory properties that lead to neuroinflammation and neurotoxicity (Dammann & O'Shea, 2008). There is also evidence of the involvement of inflammatory proteins in neurodevelopment. As discussed previously, both activated glial cells and infiltrating inflammatory cells can produce inflammatory mediators in the brain (Rocha-Ferreira & Hristova, 2015).

Inflammatory mediators in brain injury

All inflammatory biomarkers can be involved in neuroinflammation and brain damage, directly or indirectly. Proteins that recruit antigen-presenting cells (APCs), the APC membrane recruitment protein (AMER), and the complement components of the classical and alternative pathways, including complement 5 (C5), complement (C9), and complement factor B (CFB), are regulators of immune reaction and participate in the cell killing process. Also, Mannan-binding lectin serine protease 2 (MASP2), but the lectin pathway has been implicated in secondary hypoxic brain injury following traumatic brain injury (TBI) (Ciechanowska et al., 2020). In our proteomic study on asphyxiated infants (*paper IV)* several of these immunological proteins were found in increased concentrations in CSF of infants with brain injury following birth asphyxia and correlated with adverse neurodevelopmental outcome.

Cytokines are signaling molecules of the inflammatory response to either infection or hypoxia-ischemia or both. Most cytokines have both pro-and anti-inflammatory functions. In the context of a perinatal brain injury, some cytokines have mostly pro-inflammatory properties and contribute to the injurious process, whereas others have mostly anti-inflammatory properties and regulate the immune process, thereby ameliorating the injury. Evidence from several studies indicates a role of Interleukin-6 (IL-6) in relation to perinatal brain injury. It has pluripotent functions, both neurotoxic and neuroprotective. IL-6 is neuroprotective against N-methyl-D-aspartate (NMDA)-induced excitotoxicity, in vitro (Fang, X.X. 2013) and ischemic brain injury was reduced after injecting IL-6 into the brain (Suzuki, S., 2009). Nevertheless, an IL-6 over-expression in a mice modeles correlates with severe neurological symptoms (Campbell et al., 1997). It has been suggested that IL-6 may exhibit destructive functions early after hypoxic-ischemic insult, while enhancing repair and regeneration later, in the prolonged chronic phase (Jenkins et al., 2012).

Increased IL-6 concentrations in serum and/or CSF correlate with the severity of brain injury and the outcome in the HIE (Orrock et al., 2016). This was confirmed in *paper III* of this thesis (Leifsdottir et al., 2018).

Neural cell adhesion molecules (CAMs), including selectins, integrins, and immunoglobulins, are located on the surfaces of cells and participate in the binding with other cells. These molecules have an important role in brain injury as they increase cellular adhesion and infiltration of immune cells into the brain and thereby can exacerbate neuroinflammation. They are also important in brain development as they allow neural cell interactions that are

essential for neuronal migration and axon guidance, as well as synaptogenesis (Rubenstein, 2011). In *paper IV,* increased levels of several CAM proteins were found in CSF from infants with HIE and adverse neurodevelopmental outcomes. In *paper V*, higher levels were found in CSF of preterm infants than in CSF of term infants.

Prostaglandins (PGs) are lipid compounds that derive from hydrolyzed arachidonic acid (AA). Activated PGs in the brain induce symptoms of illness, like fever and pain. The cyclooxygenase (COX) isozymes convert AA to the prostanoid PGH_2 which is subsequently converted to prostanoid subclasses.

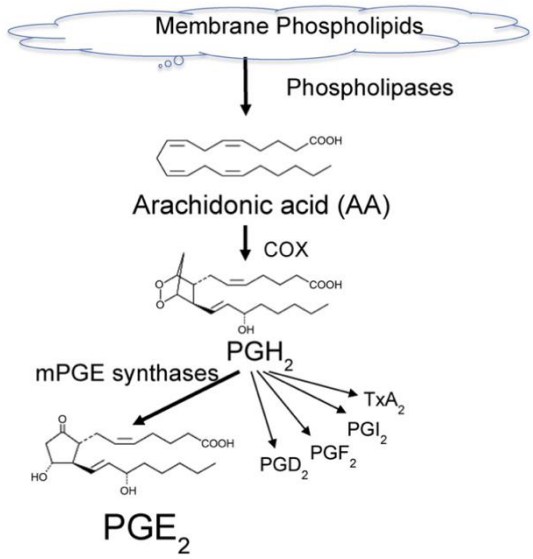

Figure 5. The conversion of arachidonic acid (AA) to prostanoid subclasses.
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Emerging data suggest that the prostanoid PGE_2 contributes to the pathophysiology of brain ischemia (Kawano et al., 2006). In *study I* we showed the PGE_2 is released into CSF following hypoxic insult and correlates with the degree of birth asphyxia. PGE_2 can act on four different subtypes of the E-prostanoid receptor. It has stimulatory or inhibitory effects depending on the receptor it binds to (Andreasson, 2010). In the ischemic brain it is involved in BBB disruption through E-prostanoid receptor subtype 2 (EP2R) on microglia and mediates neurotoxicity by binding to E-prostanoid receptor subtype 1 (EP1R) on neurons (Candelario-Jalil et al., 2007; Kawano et al.,

2006). Induced PGE2 production in the brain can be enhanced by circulating cytokines that stimulate COX isozymes and microsomal prostaglandin E synthase-1 (mPGE-1), located on BBB. Thus, an induced PGE_2 production in the brain is enhanced by system inflammatory activation and mediated by cytokines through BBB. This makes PGE_2 an important mediator of neuroinflammatory response in neonatal hypoxia-ischemia and an important messenger in the interaction between the peripheral immune mediators and the brain (Thornton et al., 2012). Evidence from studies in neonatal hypoxic brain injury models suggests brain sensitizing effects of cytokines through PGE_2 (Rummel, Matsumura, & Luheshi, 2011).

PGE_2 is involved in fetal and neonatal respiratory control through E-prostanoid receptor subtype 3 (EP3R) in the brain stem (Ek, Arias, Sawchenko, & Ericsson-Dahlstrand, 2000; Hofstetter, Saha, Siljehav, Jakobsson, & Herlenius, 2007). Stimulation of EP3R depresses breathing activity through a decrease in cAMP, which inhibits the respiration rhythm generating brainstem neurons (Ballanyi, Lalley, Hoch, & Richter, 1997). Apnea is a common side effect of PGE_2 therapy. Apneic episodes are also common and important early symptoms of infection in neonates, especially in preterm infants. In experimental studies a link has been observed between circulating interleukin-1beta (IL-1β) and depressed brainstem respiratory control through induced brain PGE_2 production in both hypoxia and infection (A. O. Hofstetter et al., 2007; Olsson, Kayhan, Lagercrantz, & Herlenius, 2003). Hypoxia can activate mPGES-1, which may induce a PGE_2 release in brainstem areas and lead to apneas. This has been shown to decrease the effect of resuscitation during a hypoxic event (Herlenius, 2011). In *study II* of this thesis, we found that increased PGE_2 levels correlated with apnea during invasive infections in human infants (Siljehav, Hofstetter, Leifsdottir, & Herlenius, 2015). These findings are important as apnea is not only a common symptom in septic infants but also one of the most frequent medical concerns in preterm infants (Finer, Higgins, Kattwinkel, & Martin, 2006). Treating preterm apnea with the adenosine receptor antagonist caffeine improves the neurodevelopmental outcome (Yang et al., 2021).

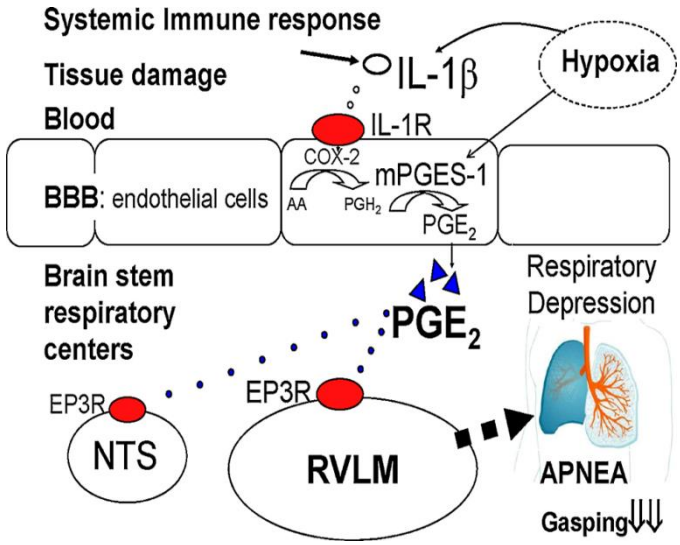

Figure 6. IL-1β induced apnea via PGE2 mediated pathway.
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Inflammatory mediators in neurodevelopment

In our proteomic study on preterm infants (*paper V)* an upregulation of inflammatory proteins was observed compared with term infants, regardless of the blood or CSF culture results. Components of the complement system are described above as contributors to tissue damage. Notably, they also have important roles in brain development and altered neuroplasticity following postnatal insults (Magdalon et al., 2020). C5 is associated with the proliferation of neural cells and the neuroprotection (Biggins et al., 2017; Coulthard et al., 2017). MASP2 is essential for the neural migration (Magdalon et al., 2020). Vascular cell adhesion molecule 1 (VCAM) enhances the proliferation of neural stem cells and the lack of VCAM leads to neural cell dysmaturation (Hu et al., 2017). Down syndrome cell adhesion molecule (DSCAM) is an immune regulatory protein. In addition to its innate immune functions, it has a key role in the synapse formation (Ly et al., 2008).

1.5 Histological and clinical presentations

The histological patterns of brain injury determine the presentation of neurodevelopmental deficits and impairments. The brain has a selective regional vulnerability depending on the developmental stage at the time of the insult. Early identification of the histological patterns is challenging and hence the clinical evaluation. This makes the discovery of biomarkers of uttermost importance.

1.5.1 Hypoxic-ischemic encephalopathy (HIE)

HIE refers to neonatal encephalopathy (NE) in term infants where hypoxia-ischemia around birth contributes to the pathology. These criteria include markers of fetal and postnatal distress along with clinical signs of neurological dysfunction or encephalopathy (Levene, Sands, Grindulis, & Moore, 1986). Therapeutic hypothermia is applied if at least one sign of perinatal hypoxia-ischemia, indicated as postnatal distress, is present and the criteria for probable moderate or severe HIE are met (Table 2).

Table 2. Criteria for the definition of HIE that needs treatment

1. Postnatal distress

- Depressed APGAR score: < 5 at 10 minutes
- Depressed pH and BE in cord blood or from infant within an hour from birth: pH <7.0 or BE -12 or lower
- Resuscitation for >10 minutes

2. Clinical signs of encephalopathy

Seizures or any two of:

- Level of conciousness: Lethargy or coma
- Spontaneous activity: Decreased
- Posture: Distal flexion, extension, decerebrate
- Primitive reflexes: Weak or absent
- Autonomic system: Abnormal pupils, bradycardia, irregular breathing

By scoring the clinical symptoms according to the Sarnat scale that was introduced in 1976 (Sarnat & Sarnat, 1976) we make clinical assessments of

infants with HIE. This scoring system has value in predicting the neurological outcome. The symptoms that are scored include the mental state, function of primary reflexes, muscle tone, and seizures, see Table 3.

Table 3. A clinical grading system for classification of HIE

	HIE-I	HIE-II	HIE-III
Level of consciousness	Hyperalert	Lethargic	Stuporous
Neuromuscular control			
Muscle tone	Normal	Mild hypotonia	Flaccid
Posture	Mild distal flexion	Strong distal flexion	Intermittent decerebration
Stretch reflexes	Overactive	Overactive	Decreased or absent
Myoclonus	Present	Present	Absent
Complex reflexes			
Suck	Weak	Weak or absent	Absent
Moro	Strong	Weak	Absent
Oculovestibular	Normal	Overactive	Weak or absent
Tonic neck	Slight	Strong	Absent
Autonomic function			
Pupils	Mydriasis	Miosis	Variable
Heart rate	Tachycardia	Bradycardia	Variable
Bronchial and salivary secretions	Sparse	Profuse	Variable
Gastrointestinal motility	Normal or decreased	Increased; diarrhea	Variable
Seizures	None	Common	Uncommon (excluding decerebration)

Modified from (Sarnat & Sarnat, 1976)

Mild HIE (stage 1) has been perceived having a good prognosis until recently that this perception has been challenged (Conway, Walsh, Boylan, & Murray, 2018). Murray and colleges described that compared with healthy controls infants with mild HIE had lower IQ scores and the cognitive measures did not differ from patients with moderate HIE (stage 2) (Murray, O'Connor, Ryan, Korotchikova, & Boylan, 2016). A recent study by Finder and colleges showing a two year follow-up on HIE infants, these findings were confirmed (Finder et al., 2020). Infants with moderate and severe HIE (stage 2 and 3) receive TH, but not infants with mild HIE (stage 1). This has been up for a debate recently and it is possible that the indications for TH will change in the near future (El-Dib et al., 2019; Sabir et al., 2021). HIE stages 2 and 3 have been correlated with motor, conitive and behvioral adversities (Edwards et al., 2010). The symptoms of neurodevelopmental deficiencies in HIE are discussed below in relation to the histological patterns.

1.5.2 Encephalopathy of prematurity

Encephalopathy of the preterm infant describes a brain disorder that results from multiple grey and white matter lesions. Subsequently multiple dysmaturational events occur, that can evolve over a prolonged period (Boardman et al., 2010; Volpe, 2019a). White matter injury is most often the initial injury that subsequently leads to dysmaturational events, like disrupted cortical microstructure and structural networks. Prematurity is the most common cause of chronic neurodevelopmental impairments. Although the overall risk of destructive lesions like cystic periventricular leukomalacia and intraventricular haemorrhage has remained stationary or declined, widespread disturbances in brain maturation rice to a wide spectrum of motor and cognitive disabilities, even with mild cerebral injury (Back, 2015).

1.5.3 Motor impairments

Neurons are the primary victims of hypoxia-ischemia, with basal ganglia and thalamus (BGT) as well as the somatosensory cortex selectively vulnerable regions and the most prevalent focus of injury (Hagberg et al., 2015). As discussed before in the developmental chapter, neuronal circuit exists between BGT and the somatosensory cortex. The active glutamatergic neurons that make the circuit connections are vulnerable to hypoxic insult (S. P. Miller et al., 2005). Neuronal deficits in this area may lead to motor disability including CP, which is the most recognized sequelae of HI brain injury (Martinez-Biarge et al., 2011). Most term infants with severe BGT lesions following perinatal asphyxia develop secondary microcephaly with a reduction in white matter that appears to be a secondary process (Millar et al., 2017).

Injury to the cerebral white matter, and a variety of neuronal-axonal disturbances, although also seen in term infants with HIE, are more characteristic of brain damage in preterm infants (A. M. Li et al., 2009). In the preterm brain, glial cells are vulnerable, especially oligodendrocytes precursor cells in the corticospinal tracts. This can result in WMI, with a high risk of motor impairments (Thompson et al., 2020). Improvements in neonatology and the care of preterm infants have led to reduced severity of WMI. The prevalence of CP has been declining across Europe over the last decades (Sellier et al., 2016). Diffuse WMI which is without focal lesions in brain tissue is observed in 50-70% of extremely preterm infants (Ment, Hirtz, & Huppi, 2009). The incidence of CP in this population has remained stationary around 10%, with that of moderate to severe CP of 3-4% (Serenius et al., 2016; Vincer, Allen, Allen, Baskett, & O'Connell, 2014).

1.5.4 Cognitive, psychiatric, and behavioral difficulties

Cortical injuries, especially in the watershed areas, are common in HIE and are strong risk factors for deficits in the cognitive development (Steinman et al., 2009). The pattern is determined by the intensity and duration of the HI insult. While BGT lesions are mostly associated with acute and profound hypoxia-ischemia, watershed cortical lesions are seen more frequently when the insult is partial and prolonged (Koehler, Yang, Lee, & Martin, 2018). Hippocampus is vulnerable to hypoxia in the early postnatal period when hippocampal neurogenesis and the formation of neural connectivity is active (Morales et al., 2008). Damage to the hippocampal area has been linked to attention deficit disorder, memory dysfunction, autism, and schizophrenia (de Haan et al., 2006; Simola et al., 2008). The mammillary bodies are also important for memory function and can be affected in HIE. A recent study describes neurocognitive and memory dysfunctions in 10 year old children who had HIE at birth. A correlation was observed between the neurocognitive deficiencies and mammillary body and hippocampi atropy on MRI. These findings were not affected by hypothermia (Annink et al., 2021). Cognitive deficiencies have been described in teenagers who were diagnosed with HIE-II at birth and were without CP or other neurological disorders. The majority had definite cognitive and executive brain dysfunctions that interfered with their daily life (Lindstrom, Lagerroos, Gillberg, & Fernell, 2006). Recnet studies also show increased rates of cognitive dysfunction in infants with HIE-I (Finder et al., 2020)

In association with diffuse WMI in preterm infants, widespread disturbances in neural maturation occur with disruption in cortical microstructure and structural networks (Thompson et al., 2019). This leads to cognitive disabilities along with the motor dysfunctions (Amer et al., 2018). As many as 25–50% of the <32w preterm group have significant cognitive and neuropsychologic sequelae and in the extremely preterm group (<28w), these figures are even higher (Amer et al., 2018). There is an overall lower mean IQ in the preterm population in comparison with infants born at term age and the IQ deficits are associated with lower gestational age at birth (Kerr-Wilson, Mackay, Smith, & Pell, 2012). Furthermore, an increased risk of learning disabilities is even observed in preterm infants with normal IQ. This is due to dysfunction in the core cognitive executive functions of the brain (Hutchinson et al., 2013). These functions are thought to underly much of the neuropsychiatric problems in this population, which is a challenge that is greatly increased in extremely preterm infants (Mulder, Pitchford, & Marlow, 2011; Vanes et al., 2021). Studies in middle childhood and adolescence have

revealed a specific behavioral phenotype of preterm infants. This phenotype consists of anxiety, attention deficiency and behavioral difficulties (Johnson & Marlow, 2011). These characteristics are also confirmed in former extreme preterm infants with psychiatric disorders. Furthermore, these children have difficulties with social interactions and communication. Preterm infants are in increased risk of autism spectrum disorders (ASD) (Agrawal, Rao, Bulsara, & Patole, 2018; Soul & Spence, 2020).

1.6 Neuroprotective approaches

Perinatal brain injury is a serious burden world wide. It carries a high risk of lifetime neurological disabilities, which highlightes the importance of discovering neuroprotective therapies. Therapeutic hypothermia (TH) is at present the only recommended treatment. It is applied to term born infants diagnosed with moderate to severe HIE after birth asphyxia (M. A. Tagin, Woolcott, Vincer, Whyte, & Stinson, 2012), but it is not yet considered safe for preterm infants (Austin, 2018). Hypothermia reduces mortality and leads to persisting improvements in outcomes of HIE (Shankaran, Laptook, Poole, & Eunice Kennedy Shriver, 2010). Nevertheless, death and disability remain a common feature in HIE (Shankaran, 2012a). Unfortunately, there are still very few complementary neuroprotective treatment options available for HIE, although experimental research has suggested many candidates, including anti-inflammatory agents, EAA antagonists, antiapoptotic agents and free radical scavengers. The safety of most of these compounds for neonates remains to be established as well as the right timing and length of treatment. No specific drugs have been established for treatment or prevention of preterm or term brain injury, although magnesium is now recommended to mothers before preterm birth as a potential brain protection for the preterm infants. Other treatments are still under investigation (McNally & Soul, 2019; Ophelders et al., 2020).

1.6.1 Therapeutic Hypothermia (TH)

It is well established that TH is neuroprotective in animal HI models (Gunn, Gunn, de Haan, Williams, & Gluckman, 1997), in cardiac arrest (Hakim, Ammar, & Reyad, 2018), stroke or spinal cord injury (Karnatovskaia, Wartenberg, & Freeman, 2014), in adult brain trauma (Leng, 2017) and in neonatal HIE (Shah, 2010). It involves cooling the infant to 33° to 34°C for 72 hours. (Wassink et al., 2019). In term infants with moderate to severe HIE it is preferably started within six hours after birth in attempt to interrupt the cascade leading to secondary energy failure. Cooling below 33°C or for a

longer duration does not enhance the neuroprotection and some studies have even showed worse outcomes (Shah, 2010; Shankaran et al., 2014). However, applying TH early, within 3 hours from birth, improves the neuroprotection, especially the motor outcomes (Thoresen et al., 2013). TH is applied in cases of moderate and severe HIE but not in mild HIE. There are insufficient evidence of the efficacy of TH in this group yet, and there is not enough known about the safety (Sabir et al., 2021). On the other hand, recommendations will be reviewed soon as there are 3 randomized studies ongoing; The COMET study, the TIME study, and the COOL PRIME study (Sabir et al., 2021).

Hypothermia decreases excitotoxicity, inhibits apoptosis, and ameliorates inflammation (Hagberg et al., 2015; Wassink et al., 2014). Decreased pro-inflammatory and increased anti-inflammatory cytokine expression has been observed in pre-clinical and clinical hypothermia studies (C. Hofstetter et al., 2007; Jenkins et al., 2012). However, diminished expression of anti-inflammatory cytokines has also been observed, (Huet et al., 2007) and interestingly not all pro-inflammatory cytokine levels are affected equally by hypothermia (Chalak et al., 2014). Furthermore, a sudden increase of proinflammatory mediators has been observed in cerebral HIE following rewarming after TH in animal models (Rocha-Ferreira et al., 2017). The role of TH in the modulation of neuroinflammation needs to be considered in the process of designing neuroprotective interventions with immunomodulatory therapeutic possibilities as the target points. Furthermore, whether TH is neuroprotective in the presence of infection sensitization is still and unanswered question, although it is likely pathogen dependent (Falck et al., 2017; Sabir et al., 2021).

Therapeutic hypothermia offers hope for asphyxiated infants, but it does certainly not protect or repair all perinatal brain injuries (K. Martinello, Hart, Yap, Mitra, & Robertson, 2017). A reduction in combined rate of mortality and severe disability was established in 18-months assessment (Edwards et al., 2010; M. A. Tagin et al., 2012). Series of completed multicenter trials showed that for infants with HIE grade 2 and 3 TH treatment doubled the probability of normal outcome (Azzopardi et al., 2014; Shankaran, 2012b). Nevertheless, although meta-analysis of trial data applies compelling evidence of the benefit of TH, the rates of death and disabilities remain as high as 45-50% (M. A. Tagin et al., 2012). This highlights the need for complementary therapies.

Some indications of beneficial effects of hypothermia for preterm infants are seen in animal studies (Higgins & Shankaran, 2011). Nevertheless, it has not proven to be a safe treatment for this patient group. There have been

concerning outcomes in small pilot studies (Rao et al., 2017). Preterm infants may have vulnerabilities that increase the likelihood of complications (Herrera et al., 2018).

1.6.2 How biochemical biomarkers may lead to novel therapeutic options

Finding ways to identify complementary therapies in combination with therapeutic hypothermia to further improve neuroprotection is a challenge for the future. Biomarkers reflecting the pathophysiology of the evolving brain injury could allow us to identify infants who still benefit from intervention and aid in the development of more targeted treatments.

In the studies of the present thesis many of the CSF samples were gathered after 6 hours, whereby hypothermia is initiated today. Therefore the results may not accurately identify the infants who would benefit from therapeutic hypothermia. Nevertheless, the observed biomarkers may enhance our knowledge of the pathophysiology and help identify novel complementary therapeutic options. Many were related to neurodevelopment and neuroinflammation, and thus might lead to a better understanding of the pathophysiology of the tertiary phase of brain injury. This is an important venue of additional therapy.

Figure 7 describes the pathophysiological timeline in HI brain injury and known biomarkers that have been correlated with HIE in previous studies as well as some of the proteins observed in our studies. Acknowledging this timeline is helpful when considering complementary neuroprotective strategies in HIE. In the same figure, are few of the neuroprotective therapies that are now under investigation.

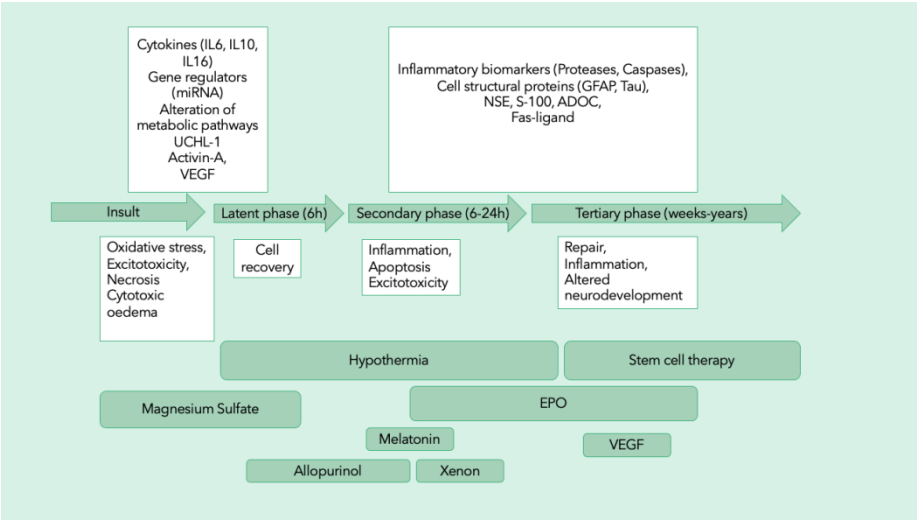

Figure 7. Biomarkers in HIE

No single biomarker is robust enough to diagnose the brain injury accurately or predict outcome, but many are promising and under investigation. The early detection and assessment of the degree of neonatal HIE is highly important as it influences therapeutic intervention with hypothermia, which is applied before 6 hours from birth. Using the current clinical criteria approximately 20% of the infants who develop adverse neurological outcome are assigned a mild grade and do not receive hypothermic treatment (DuPont et al., 2013). Early biomarkers with the potential to make the diagnosis more accurate would clearly be of benefit for these infants. The inflammatory proteins IL-6, IL-10 and IL-16 are early biomarkers with the ability to predict severe outcome in HIE (Ahearne, Chang, Walsh, Boylan, & Murray, 2017; Massaro et al., 2018). Decrased levels of vascular endothelial growth factor (VEGF) and increased levels of Activin-A are also indicative of early HI brain injury and delayed treatment with VEGF enhances recovery (Florio et al., 2010; J. Li et al., 2020). Ubiquitin C-terminal hydrolase L1 (UCHL1) is a major protein component of the neuronal cytoplasm and currently developed as a biomarker of traumatic brain injury in adults (Papa et al., 2017). It is elevated in cord blood following neonatal HI brain injury (Chalak et al., 2014).

Glial fibrillary acidic protein is a cytoskeletal protein in astrocytes and one of the most promising biomarkers for predicting the outome in HIE. It has been correlated with severe outcome and abnormal MRI findings (Blennow, Hagberg, & Rosengren, 1995; Chalak et al., 2014). Tau is another cytoskeletal protein, a known biomarker in Alzheimer's and Parkinson's

disease and raised levels have been implicated in HIE (Massaro et al., 2018; Takahashi et al., 2014).

Neuron specific enolase (NSE) which is involved in glycolytic energy metabolism has been correlated with the risk of death or severe neurological impairment in HIE (Roka et al., 2012). Aldolase C (ALDOC), is a protein of metabolism in astrocytes and released when there is an astrocyte injury. It has been correlated with hypoxic ischemia after traumatic brain injury in animal models (Thelin et al., 2018). These were among the proteins that were found in increased levels of HIE infants with adverse outcome compared with control infants in *study IV*.

1.6.3 Other treatment strategies

Several studies are exploring possible candidates that may offer further neuroprotection in term and preterm infants. In pre-clinical models of hypoxic brain injury, the focus is generally on slowing down the machinery of the injury cascades. This includes decreasing oxidative injury and apoptosis, antagonizing excitatory neurotransmitters, inhibiting the release of certain pro-inflammatory molecules and/or blocking receptors (Chakkarapani et al., 2021; K. H. Cho, Davidson, Dean, Bennet, & Gunn, 2020), see Figure 8. The first line in Figure 8 displays the injurious processes and above are the possible applicable treatment strategies. Many treatments relate to more than one injurious process.

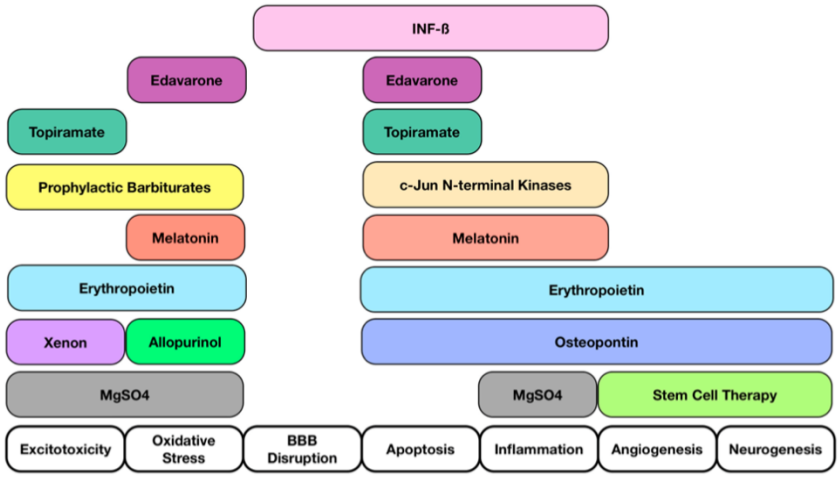

Figure 8. Potential neuroprotective agents in perinatal brain injury.
Ref. Dixon, B.J., et al., Int J Mol Sci. 2015;16(9):22368-401.

Several compounds have been observed that have beneficial effects on neuronal repair and regeneration, enhance cell growth and differentiation as well as long-term integration into neural networks. These include agents like growth factors and stem cells (Moral, Robertson, Goni-de-Cerio, & Alonso-Alconada, 2019). Several therapies that offer neuroprotection are currently being explored in preclinical studies, or in clinical trials at phase 2 and 3. Some of these agents and its correlations are discussed here below.

Reducing excitotoxicity

Blocking NMDA and AMPA receptors reduces neuronal damage in experimental HI models (Hagberg, Gilland, Diemer, & Andine, 1994).

Topiramate is an clinically available anticonvulsant. It is protective against white matter injury in rodent model (Sfaello, Baud, Arzimanoglou, & Gressens, 2005). The administration of Topiramate in neonates with HIE is safe but the efficacy of neuroprotection has not been demonstrated yet in clinical studies (Filippi et al., 2018).

Cannabidiol (CBD) reduces rodent brain injury short-term when administered after HI (Fernandez-Lopez et al., 2007). More recently it has been shown to enhance the benefits of hypothermia early in HI brain injury (Lafuente et al., 2016).

Xenon is a monoatomic gas which appears to have neuroprotective effects, mainly as a potent NMDA receptor antagonist, but it also activates antiapoptotic proteins (Nair & Kumar, 2018). A randomized controlled clinical trial in 92 infants with moderate to severe HIE (The TOBY-Xe trial) showed no additional benefit of Xenon treatment in combination with TH (Azzopardi et al., 2016). However, a recent Cochrane review concludes that the data are inconclusive as to whether Xenon has neuroprotective effects in neonates (Ruegger, Davis, & Cheong, 2018).

Neuroserpin is an enzyme secreted by neurons. It is found in excess amount in the subplate of neonatal mouse brain and is important in cortical neuroplasticity (Kondo, Al-Hasani, Hoerder-Suabedissen, Wang, & Molnar, 2015). It has been suggested as potential therapy in HI brain injury (Millar et al., 2017).

Reducing oxidative stress

Allopurinol is a free radical scavenger. It reduces delayed cell death (Annink et al., 2017) and clinical studies have shown improved long-term neurodevelopmental outcomes in infants with moderate HIE (Kaandorp et al.,

2012). However, the window of benefit is only brief which makes it difficult to use in human clinical settings (Benders et al., 2006).

Melatonin has diverse anti-oxidative mechanisms, protects against excitotoxic damage, and exerts anti-inflammatory and antiapoptotic functions (Juul & Ferriero, 2014). It improved cerebral protection when applied in combination with TH in newborn piglets with HI brain injury (Robertson et al., 2020) and beneficial effects were also described in a small clinical pilot study (Aly et al., 2015). A recent systematic review described limitations to the trials performed so far (Ahmed, Pullattayil, Robertson, & More, 2021).

Curcumin is an antioxidant with anti-inflammatory activity. Curcumin has been found to reduce grey and white matter tissue loss in neonatal mice (Rocha-Ferreira et al., 2019). No clinical studies are available.

Anti-inflammatory agents

Inflammation contributes to injury in the developing brain. Therefore agents that target the neuroinflammatory pathways are tested in animal models (L. Wu et al., 2020). As inflammation is associated with the prolonged chronic phase of the brain injury, therapies may need to be provided during a long period (Fleiss & Gressens, 2012).

Minocycline is a derivative of tetracycline and has neuroprotective properties in hypoxic brain injury in animal HI models, through its' abilities to suppress microglial activation (Lechpammer et al., 2008). Notably, not all studies have reported beneficial effects of minocycline and an exacerbation of HI brain injury was described in a pre-clinical model (M. Tsuji, Wilson, Lange, & Johnston, 2004).

Toll-like receptor agonists reduce HI brain injury via preconditioning or tolerance (Dhillon et al., 2015). It also induces anti-inflammatory cytokine expression from microglia (Lobo-Silva, Carriche, Castro, Roque, & Saraiva, 2017). Administration of Toll-like receptor agonist improved neurological outcome following asphyxia in HI animal model (K. H. T. Cho et al., 2019; Ye et al., 2018). Further studies are needed to assess safety and efficacy.

Stem cells have strong neuroprotective capacities through modulation of inflammation. They increase cell survival and repair (van Velthoven, Kavelaars, van Bel, & Heijnen, 2010). At 3-10 days after HI brain insult, intranasal or intracerebral treatment with mesenchymal stem cells reduced brain injury and improved memory functions in a rodent neonatal model (Donega et al., 2013). Also, a significant improvement of oligodendrocyte maturation and reduced neuronal loss was found in stem cell treated fetal sheep asphyxia models (van den Heuij et al., 2019). Mesenchymal stem cell therapy combined with hypothermia improved outcome in a pre-clinical model

(Park et al., 2015). In a recent piglet model stem cell therapy in combination with hypothermia augmented brain protection compared with hypothermia alone (Robertson et al., 2021). Nevertheless, safety and efficacy must be assessed further. There have been indications of adverse effects with exacerbation of HI brain injury with stem cell therapy in some studies (Herz et al., 2018). Human trials are at a very early stage. A phase I trial with cord derived allogenic stem cells in neonates with HIE has been completed but the results not yet reported (Chakkarapani et al., 2021).

Growth factors

Increased expression of growth factors is a part of the endogenous response following hypoxia-ischemia in the brain. Growth factors are important in normal brain development and function but following injury they may mediate changes in apoptosis and inflammation in addition to neurotrophic properties. They are considered attractive therapeutic candidates and have been tested in a multitude of brain injury or neurological disease models (Larpthaveesarp, Ferriero, & Gonzalez, 2015).

Erythropoietin (EPO) is an promising neuroprotective substance with anti-apoptotic, anti-inflammatory, and anti-excitotoxic effects (Huang et al., 2019; Oorschot, Sizemore, & Amer, 2020). An experimental study found that EPO reduces apoptotic cell death though reduction in Fas and Fas-ligand expression in the brain of neonatal rats with HI brain injury (Huang et al., 2019). Concurrent EPO and TH treatment in neonatal asphyxia improves outcome more than TH treatment alone. This has been shown in preclinical and clinical studies, including a multicentre Phase II study (Mulkey et al., 2017; Traudt et al., 2013). A recent meta-analysis from six randomized controlled trials and a systematic review concluded that EPO treatment is neuroprotective in HIE (Razak & Hussain, 2019). A Phase III multicentre placebo controlled randomised trial is now underway, evaluating the effect of high-dose EPO in combination with TH in neonates with HIE: “High-Dose Erythropoietin for Asphyxia and Encephalopathy” (HEAL) (Juul et al., 2018). The estimated completion of that study is in September 2022. In Australia, another, similar, Phase III trial is ongoing: the “Preventing Adverse Outcomes of Neonatal HIE with Erythropoietin” (PAEAN) trial. The effectiveness of EPO as monotherapy in neonatal asphyxia in low resource countries where HT is unavailable has been established. In this group, death or disability was 70% in untreated infants but 40% in infants receiving EPO treatment (Malla, Asimi, Teli, Shaheen, & Bhat, 2017). A recent metaanalysis of a pooled data confirms this (Ivain et al., 2021).

However, the evidence on the efficacy of EPO in HI brain injury is still unclear and there are existing concerns. Some suggest that the neuroprotective and repair mechanisms of EPO overlap with those of hypothermia and thus, is not an effective adjunct therapy (Lv et al., 2017). A recent study in fetal-sheep HIE model in New-Zealand showed no benefit from combining EPO with therapeutic hypothermia, assessed with physiological parameters and histology (Wassink et al., 2020). EPO application has even been found to worsen the injury in situations of extreme oxidative stress (Sheldon, Windsor, Lee, Arteaga Cabeza, & Ferriero, 2017).

EPO has been shown to prevent preterm birth and increase survival in the offspring (J. Zhang et al., 2020). A significantly decreased rates of IVH and periventricular leukomalacia was observed in a Cochrane review of 34 studies on EPO administration to preterm infants (Ohlsson & Aher, 2020). But results of the effect on long-term neurological outcome are conflicting (Fauchere et al., 2015; Song et al., 2016). In a recent phase III Preterm Erythropoietin Neuroprotection (PENUT) randomized placebo controlled clinical trial with high-dose EPO treatment in extremely preterm infants it did not lower the risk of neurodevelopmental impairment (Juul et al., 2020).

Insulin-like growth factor-1 (IGF-1) promotes angiogenesis, neurogenesis and myelination in the developing brain (Russo, Gluckman, Feldman, & Werther, 2005). Low endogenous IGF-1 levels have been observed in extremely preterm infants correlated with increased risk for ROP, neurodevelopmental impairments and other complications of prematurity (Hellstrom et al., 2003; Lofqvist et al., 2006). A Phase II randomized controlled trial was conducted, administrating IGF-1 to a group of extreme preterm infants. No effect was observed on the incidence of ROP but a trend to decreased incidence of IVH was noted (Ley et al., 2019).

Exendin-4 is a medication for type-2 diabetes but is also neuroprotective (King et al., 2020). From preclinical and clinical data Exendin-4 is considered a promising candidate for the treatment of neurodegenerative diseases (Athauda & Foltynie, 2018). In a recent study, exendin-4 was found to be highly neuroprotective treatment of neonatal HIE in a mouse model and when used in synergy with hypothermia the therapeutic efficacy was enhanced (Rocha-Ferreira et al., 2018).

Metformin is another type-2 diabetes medication, considered to be a promising candidate for treatment in HI brain injury (Disdier & Stonestreet, 2020). It attenuates HI brain injury through inhibiting apoptosis and ameliorating neuroinflammation (M. Fang et al., 2017).

Vascular endothelial growth factors (VEGFs) are important growth factors for angiogenesis and neurogenesis. In experimental studies they

improve neuronal viability and function and ameliorate cognitive impairment (Guo et al., 2016). Nevertheless, deleterious effects have been reported. Early administration of VEGF antagonist like Curcumin, decreases infarct volume in preclinical studies (J. Li et al., 2020). Anti-VEGF has beneficial effects in retinopathy of prematurity (Tran, Cernichiaro-Espinosa, & Berrocal, 2018). Recently the administration of VEGFC was shown to improve neurological repair following traumatic brain injury in a rat model through modulating microglial activation (Ju, Xu, Wang, & Zhang, 2019). In *study V* of our thesis lower CSF levels correlated with adverse neurological outcome in preterm infants.

Magnesium Sulphate (MgSO4) administered to mothers antenatally immediately prior to preterm birth decreases mortality and exerts neuroprotective effects for the preterm infant (Koning et al., 2018). Meta-analysis demonstrated a reduction in the incidence of CP or other motor disabilities by 30% (Doyle, Crowther, Middleton, Marret, & Rouse, 2009). Randomized controlled trials have confirmed this and additionally showed a diminished risk of lesions on brain ultrasound (Gano et al., 2016; Hirtz et al., 2015). A recent multicenter diffusion tensor imaging cohort study on preterm infants in New Zealand and Australia (the MagNUM study) reported an association of antenatal MgSO4 administration with enhanced white matter development (Poppe et al., 2020). Gene upregulation is thought be an important contributor to the beneficial mechanisms of MgSO4. This might explain the neuroprotective effects in the preterm infants when administrated to the mothers antenatally (Koning et al., 2019).

In the full-term population there is less evidence on the benefits of MgSO4 although experimental studies have demonstrated reduced apoptosis in HI brain injury (Turkyilmaz, Turkyilmaz, Atalay, Soylemezoglu, & Celasun, 2002). A systematic review showed a significant improvement in short term outcome with MgSO4 treatment, but there was also a trend toward increased mortality (M. Tagin, Shah, & Lee, 2013). A recent clinical trial shows some benefit in infants with HIE who also received melatonin (El Farargy & Soliman, 2019). Further pre-clinical testing is needed, as is suggested in a recent systematic review (Galinsky et al., 2020).

1.7 Summary

A high incidence of early neonatal mortality and severe lifelong neurodevelopmental impairments originating in the neonatal period is a serious burden worldwide. Preterm birth, invasive neonatal infections and

perinatal brain insults leading to hypoxic-ischemic encephalopathy (HIE) are the most common causes.

Both the etiology and pathophysiology of perinatal brain injury are complex, and many questions are unresolved. It is likely that in many cases the causal factors are multiple rather that a single damaging event. Neuroinflammation is one of the key molecular pathways in both HIE and encephalopathy of prematurity. It may contribute to a prolonged chronic phase in HI brain injury, leading to epigenetic changes with altered brain plasticity and aberrant neurodevelopment.

The early recognition of infants at risk of serious complications is a challenge as the initial clinical signs often are of limited precision. Therapeutic hypothermia is the recommended treatment today for infants with hypoxic ischemic brain damage who are born at term age, while no treatment is available for preterm infants. Biomarkers could offer a better knowledge of the brain injury pathology and aid in the identification of adjuvant treatment options in this severe and often devastating condition. The recognition of a prolonged and persistent phase of the perinatal brain injury is important. It gives possibilities of a longer therapeutic time window as promotion of cell repair and regeneration is important in the developing brain, even long after the insult.

In the studies of this thesis, we measured proteins in CSF of term and preterm infants. The main purpose was to identify biomarkers of importance to predict the magnitude of brain injury and the clinical outcome of preterm and term infants. Also, to contribute to the understanding of the pathological processes of brain injury and brain dysmaturation in the developing brain, which is highly important and may pave the way for treatment possibilities.

2 Aims

The main purpose of this thesis was to discover reliable biomarkers in CSF to

i) Identify the degree of HIE and predict the outcome of patients following perinatal asphyxia.

ii) Identify inflammatory processes, that correlate with sepsis and cardiorespiratory disturbances.

iii) Identify preterm infants at risk of adverse neurodevelopmental outcome.

2.1 Hypotheses

The following hypotheses were put forward and consequently tested:

- An upregulation of neuroinflammation would be observed in CSF following birth asphyxia in term infants.
- Inflammatory biomarkers could serve as diagnostic tools in the early stages of HIE and predict the long-term clinical outcome.
- The inflammatory biomarker prostaglandin E_2 (PGE_2) would be released into CSF of infants with early sepsis and correlate with cardiorespiratory disturbances.
- Brain-specific biomarkers that reflect the various processes of HI brain injury would be released into CSF after hypoxic-ischaemic brain insult. These include biomarkers of energy failure, apoptosis, excitotoxicity, and inflammation as well as cytoskeletal proteins that are released upon brain cell destruction. The CSF levels of these biomarkers would correlate with the degree of HIE and the clinical outcome.
- The CSF proteome, including inflammatory, structural and functional brain antigens, would be different in preterm infants than in infants born at term age.
- Preterm infants would present a specific proteomic signature predictive of neurodevelopmental outcomes.

2.2 Specific aims

I. To evaluate the diagnostic and prognostic values of the inflammatory mediator prostaglandin E_2 (PGE_2) in CSF of infants who suffered birth asphyxia. Furthermore, to determine the advantage of analyzing the more stable derivative of PGE_2, prostaglandin E_2 metabolite (PGEM), for this purpose.

II. To determine the role of PGE_2 in apnea, bradycardia, and desaturation (ABD) events during neonatal infection and the value of PGE_2 in diagnosing early sepsis.

III. To assess the significance of interleukin-6 (IL-6) and fas-ligand (FasL) levels in CSF in correlation with the severity of HIE, and long-term outcome.

IV. To evaluate the use of targeted profiling of CSF for predicting brain injury severity and long-term outcome following birth asphyxia in term-born infants.

V. To identify, with the use of protein arrays, the CSF proteome of preterm infants in comparison with term-born infants, including structural and functional brain-specific proteins and inflammatory mediators.

VI. To identify a specific proteome in preterm infants that would predict the neurodevelopmental outcome.

3 Materials and methods

Patients and controls were recruited at the Karolinska Hospital in Stockholm between October 1999 and January 2005. All studies were approved by the regional ethics committees at the Karolinska Institute and the Stockholm County (Dnr 98-246, 2003-174, 2011/1891-31). The performance of the studies followed the principles of the declaration of Helsinki 1975 and its revision in 1983 as well as the European Community guidelines.

3.1 Patient population and clinical assessment

3.1.1 Patient population

A total of 125 patients were recruited from the NICU and general maternity neonatal ward at the Karolinska University Hospital. Of them, 111 infants were included in the four studies. The cohort was divided into three groups.

i) Term born infants with HIE after birth asphyxia (study I, III and IV).

ii) Preterm and term-born infants with apnea, bradycardia, desaturation (ABD) events, and/or suspected infection (study II and V).

iii) The term-born infants who served as controls had clinical and laboratory signs suggestive of infection. They had no suggested pathology of the brain, no apnea, bradycardia, desaturation (ABD) events, and negative cultures in blood and cerebrospinal fluid (all studies).

Patient cohort in studies I, III, and IV

Forty-four (n=44) term-born infants who met the inclusion criteria for HIE were included into study III, n=35 were included into study I and n=18 into study IV. Twenty infants who served as controls were included in studies I and III and of these 10 were in study IV.

Inclusion criteria of patients were symptoms of encephalopathy within 6 hours of life in accordance with the National Institute of Child Health and Human Development classification for modified Sarnat staging (Sarnat & Sarnat, 1976) (see table 3), in addition to signs of fetal and/or neonatal asphyxia. These criteria included signs of fetal bradycardia on cardiotocographic registration, a pH of <7.1 or a lactate of >4.8 in scalp

blood, an Apgar score under 6 at 5 min. pH of ≤7.00 and/or a base deficit of ≥16 mEq in umbilical arterial blood, or blood collected within an hour of birth, and a resuscitation for more than 3 min.

Exclusion criteria were encephalopathy unrelated to asphyxia, including congenital malformations or chromosomal abnormalities, metabolic diseases, and intrauterine or perinatal infections.

Patient cohort in studies II and V

Twenty-five (n=25) and 27 (n=27) preterm infants born from the 23rd to 34th week of gestation were enrolled prospectively into studies II and V, respectively. Twenty-two and 10 term-born infants served as controls in studies II and V, respectively. Inclusion criteria for the preterm group was suspected infection with or without ABD events. The control infants also had suspected infection, but all had negative cultures in blood and CSF. Exclusion criteria were brain pathology other than infection that can give rise to secondary apneas, like intraventricular hemorrhage more than grade 2 or periventricular leukomalacia at the time of lumbar puncture, and seizures.

Controls

Term-born infants who had clinical and laboratory signs suggestive of infection served as controls. At the time of recruitment lumbar puncture was a standard care for a child who had symptoms compatible with infection. Many of these infants never got very sick and they recovered quickly. Three of the infants in the control group were treated in a respirator. Of them two had meconium aspiration syndrom and one infant had pneumonia. The other control infants had increased CRP and respiratory symptoms but did not need ventilatory support and they recovered in few days. They had no suggested pathology of the brain, no apnea, bradycardia, desaturation (ABD) events, and negative cultures in blood and cerebrospinal fluid (all studies). All had normal outcome at 18 months.

3.1.2 Clinical assessment

Studies I, II, and IV

Before enrolling the patients into the studies, a clinical evaluation was performed. Then all enrolled infants were monitored with clinical neurological assessment at approximately 12, 36, 72 hours, and 7 days after birth. Scoring of neurological symptoms according to the Sarnat staging for HIE (see table 3) was used to classify patients into HIE stages I, II, and III. Standardized neurological assessment using the Hammersmith Neurological

Examination was performed on all controls before discharge. They all had a follow-up at outpatient pediatric care centers from where the information on outcome was gathered.

All patients received conventional treatment that was standard in most neonatal centers at the time of patient recruitment. This included fluid restriction, mechanical ventilation, inotropic support as well as anti-convulsant when needed.

Magnetic resonance imaging (MRI) was performed on four infants, who all had HIE-III. They all had profound ischemic changes in the basal ganglia and thalami on the MRI. Of these four infants, two died in the neonatal period and one survived with adverse neurological outcomes at 18 months. None of the other patients had MRI as it was not routinely performed at the time of recruitment but all patients with HIE-II and HIE-III underwent computed tomography (CT) scan. All infants with HIE-III and eight of the 10 infants with HIE-II who had an adverse neurological outcome at 18 months had signs of edema on CT. In addition, three infants with HIE-II who had a normal neurological outcome at 18 months had edema on CT.

Neurological evaluation of surviving patients was performed at 3, 6, and 18 months of age by a senior neuro pediatrician. Neurodevelopmental assessment using Bayley Scales of Infant and Toddler Development (BSID), was performed on patients who exhibited symptoms of abnormal neurology at 18 months assessment. The adverse outcome was defined as either death or unfavorable neurodevelopment at 18 months. This included CP, mental developmental index (MDI) <85, seizure disorder, deafness, and blindness.

3.1.3 Cardiorespiratory recordings

Study II

After lumbar puncture, ABD events were quantified using the KIDS event monitoring system (Hoffrichter, Schwerin, Germany). Impedance pneumography and 3-lead electrocardiogram detected chest wall movements and heart rate variability, respectively. We defined apnea or hypopnea as a reduction in respiration rate by 84% from the mean value of the previous 25 seconds, for 10 seconds or more. We defined bradycardia as a reduction in heart rate to 80 bpm or lower.

3.1.4 CSF collection

Additional CSF was collected for research purposes from infants who underwent clinically indicated lumbar puncture (LP). All samples were centrifugated for 10 minutes at 3000 revolutions per minute (rpm), and the

supernatant was stored at 80 °C until analyzed.

Study I, III, and IV

The samples were collected within 72 hours from birth. Additional samples were obtained from 4 patients in study I and 12 patients in study III. In the patients who underwent a single LP, the procedure was performed at a median time of 22.5 (interquartile range (IQR), 15–42) and for controls 26 (IQR 13.5–48) hours from birth, respectively. In the patients who underwent two LPs, the median time was 14 (IQR 8–26) and 72 (IQR 60–111) hours after birth, respectively. When two samples were available from the same patient, mean levels of the two samples were included in the analysis. In study I the median time (IQR) of CSF gathering was 20.2 (8-30) hours, in study III 22.5 (15-42) hours and in study IV 24 (12-30) hours.

Study II and V

LP was generally performed later in the preterm group, or at a median of 11 (3.5 to 19.5) postnatal days, while at median 2 postnatal days (1 to 2.5d) in term infants.

3.1.5 CSF analysis

Study I and II

Enzyme immunoassay (EIA) was used to analyze PGE2 and PGEM in the CSF samples. This is a prostaglandin E2 metabolite assay that produces a single stable derivative by conversion of PGA2 and PGE2. The calculated values of the stable derivative represented the concentrations in the samples.

We also performed standard laboratory analysis in the samples, including the red blood cell and white blood cell count. Standard biomarkers of infection, CRP, and white blood cells were measured in blood. Bacterial cultures in blood and CSF were performed.

Study III

ELISA (Diaclone Research, Besancon, France) was used to analyze IL-6, IL-6R, and FasL in all CSF samples. The detection limit for IL-6 and IL-6R was 2 pg/mL and for FasL and sFas 12 pg/mL and 47 pg/mL, respectively.

Study IV and V

In these studies, we used an antibody suspension bead array to perform protein-profiling in CSF. The arrays were based on antibodies (affinity reagents) for protein fragments, produced from protein-coding genes within

the Human Protein Atlas (HPA) (www.proteinatlas.org) (Collaborators, 2021, November 18). This was a single molecule-sensitive fluorescence-linked immunosorbent assay. Brain enriched proteins and inflammatory proteins were selected for the analysis based on association with HI brain injury and previous indications of utility as brain injury biomarkers. They represented a variety of functions, including inflammatory response, metabolic dysregulation, and structural nerve cell damage.

Protein profiling

For the microarray 220 antibodies targeting 178 unique prechosen brain enriched and inflammatory proteins were produced. The antibodies were covalently bound to magnetic color-coded beads (500 000 beads per identity, MagPlex Luminex Corp.) and the different bead identities were combined to make a suspension bead array.

The samples were then randomized onto 96-well microtiter plates and biotin (NHS-PEG4-Biotin, Thermo Scientific) was used for labeling. The interacting proteins were cross-linked in paraformaldehyde (0.4% in PBS) and a detection agent (streptavidin-conjugated R-phycoerythrin, 1:750 diluted in PBST, Invitrogen) was added. The interacting proteins were analyzed using the FlexMap3D instrument (Luminex Corp, Texas, USA), which reported the relative abundance of proteins in the samples as median fluorescent intensities (MFI).

3.1.6 Statistical methods

Analysis of clinical and laboratory data

We used descriptive methods to represent clinical and laboratory data, medians with interquartile ranges (IQR) from 25^{th} to 75^{th} percentile and means ± standard error of the mean (SEM) or 95% confidence intervals.

To analyze the differences in pH and BE between the patients and controls in *study I* we used the Dunnett's test and in *studies, I and II* student's t-test was applied to assess parametric clinical data. Levene's test was applied to test unequal variance in *study II*. When positive then Welch's test was applied on parametric data and Wilcoxon χ^2 test on nonparametric data.

Mann-Whitney U test was used to compare the independent groups in *studies III, IV, and V,* and for comparing groups consisting of categorical variables in *study III* the chi-squared test was used.

Analysis of metabolites in CSF

The levels of biomarkers in CSF were presented as mean ±SEM in *studies I and II* but as medians (IQR) in studies *III*, *IV, and V*.

To determine the correlation of PGEM levels in *study I* to the degree of HIE and outcome, Tukey-Kramer HSD (honestly significant difference) test was applied.

In *study II* an investigator who was blinded to the PGE_2 analysis performed a retrospective collection of cardiorespiratory recordings for the analysis of ABD events. The correlation analysis between PGE_2 levels and the associated variables was done using a generalized linear model. ROC curves evaluated the correlation between ABD events and PGEM levels.

In *studies III*, *IV, and V* Mann-Whitney U test was applied for comparison of two independent groups consisting of continuous variables, but Wilcoxon matched-pairs signed-rank test when the groups were related. Comparing tree independent groups consisting of continuous variables, like the outcome groups and controls, Kruskal-Wallis test with Dunn's multiple comparison post hoc test was used and if the groups were related then Friedman test with Dunn's multiple comparison post hoc test was used.

Receiver operating characteristic (ROC) curves were generated to evaluate the correlation of measured biomarkers to HIE grade and outcome in *studies I and III* as well as the correlation of ABD events and the levels of PGEM in *study II*.

Analyzing proteomic profiles

To reduce the number of dimensions of all the measured proteins principal component analysis (PCA) was performed in *study IV.* In that study, we also performed pathway analysis using the Biocarta gene set and BioGRID for the protein-protein interaction network and enrichment analysis. The threshold for inclusion in the input was $p < 0.05$.

For visualization of the results in *studies IV and V,* all the data was converted to log_2- transformed fold changes of protein levels and displayed on a Volcano plot. In those studies, scatter plots were created for visualization of big data differences in unique proteins.

Statistical software used

All statistical analysis and the graphics were performed using JMP 10 and pro15 (SAS Institute Inc., Cary, NC, USA) as well as the R, version 4.0.3, the FactoMineR package, version 1.34 (R.D.C. Team, 2010) for principal component analysis (PCA) and R package pathfinder (Ulgen, Ozisik, & Sezerman, 2019) for pathway analysis.

4 Results

4.1 Study I

Thirty-five infants, 24 patients, and 11 controls were enrolled in this study. Of the patient group, *n=8* had HIE-I, *n=9* HIE-II, and *n=7* HIE-III. Five infants died during the first days of life and seven had adverse neurological outcomes at 18 months assessment. The remaining 12 infants had normal outcomes as well as all controls.

4.1.1 PGE_2 and perinatal asphyxia

Apgar score and arterial pH were negatively correlated with PGE_2 metabolite (PGEM) levels in CSF. This relationship indicates that anoxia induces the release of PGE_2 in the brain.

4.1.2 PGE_2 and HIE degree

A positive correlation was observed between the degree of HIE and PGEM levels in CSF, which demonstrates the severity of HI brain injury (Fig. 9).

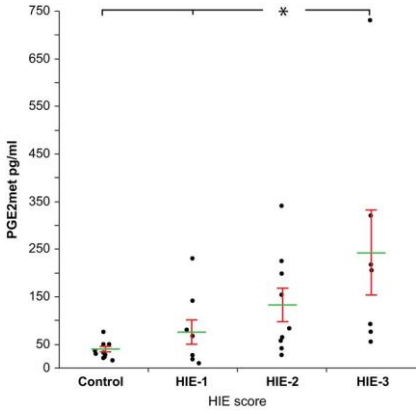

Figure 9. Correlation between PGEM and HIE grade.

4.1.3 PGE_2 levels in correlation with outcome

The outcome was defined as normal, adverse, or death. Long-term neurological outcome was according to eighteen-month assessment defined adverse in case of CP, microcephaly, cognitive developmental delay (MDI <85) or epilepsy.

Irrespective of HIE grade, patients with adverse neurological outcome on an 18-month assessment (n=7) and those who died (n=5), had higher PGEM values than controls and asphyxiated infants with normal outcome (Fig. 10, A). On a receiver operating characteristic (ROC) curve the calculated discriminative power estimated by the area under the curve (AUC) was 90% for PGEM in relation to outcome. This showed a reasonable discriminative power, with 85% sensitivity and 83% specificity at the indicated cut-off. When compared to ROC generated for pH and -BE in relation to outcome the PGEM levels had higher AUC than both. The PGE2 metabolite in CSF had a high predictive value in relation to the outcome which makes it a promising biomarker for prognosis (Fig. 10, B).

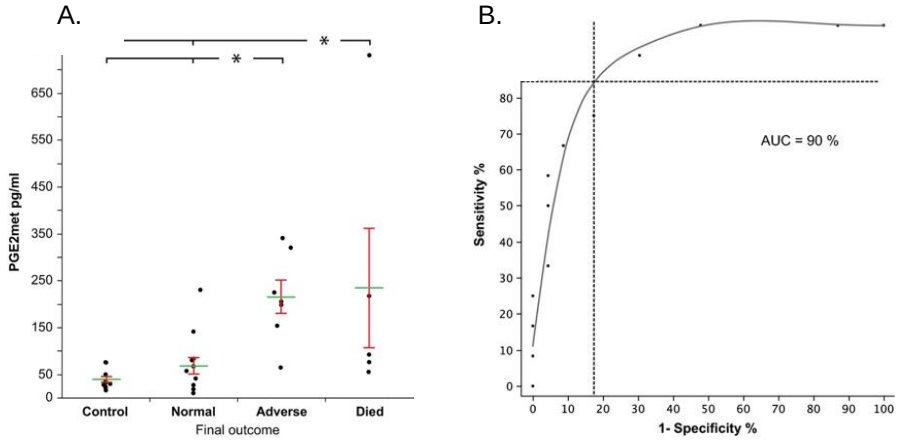

Figure 10. Correlation between PGEM and outcome (A). ROC curve showing a predictive value of PGEM in relation to outcome (B). The hoizontal green lines indicate mean values for each group and the whiskers are standard error of the mean (SEM).

Key points in paper I:

- PGE2 metabolite (PGEM) is released into CSF following asphyxia
- PGEM levels demonstrates the severity of HI brain injury
- PGEM predicts outcome with high sensitivity and specificity

4.2 Study II

In this study PGE_2 was evaluated in CSF from preterm (*n=25*) and full-term (*n=22*) infants. All infants had suspected infection. Infants with negative bacterial cultures in blood or CSF were controls in the study. Several (*n=17*) infants had apnea episodes confirmed with a noninvasive cardiorespiratory recording.

4.2.1 PGE2 levels in sepsis

Of those who had negative cultures in blood and/or CSF, PGEM levels were higher in CSF from preterm than in term infants (Fig. 11, A). Infants who had culture-verified sepsis and/or meningitis had higher PGEM concentrations in CSF than infants without culture-confirmation (Fig. 11, B).

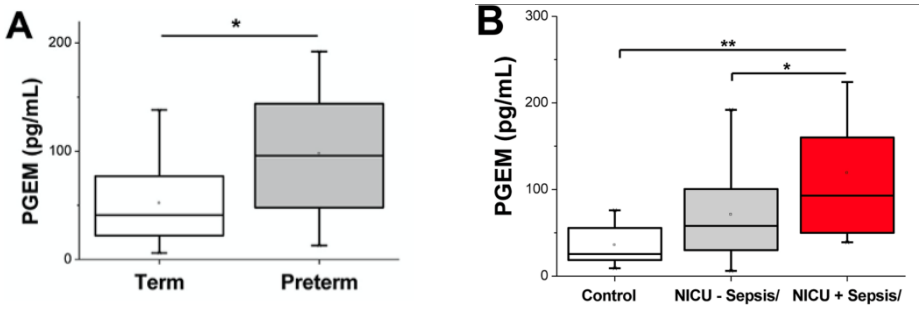

Figure 11. Comparison of PGEM levels in term and preterm infants (A). PGEM levels in correlation with culture results (B). The data values are presented as mean and standard error of the mean (SEM). *p<0.05, **p<0.01.

4.2.2 PGEM versus CRP as markers of infection

ROC curve analysis showed more specificity and sensitivity of PGEM levels in CSF than C-reactive protein (CRP) in blood, in differentiating between infants who had culture verified bacterial infections and those who didn‘t.

4.2.3 ABD events

Cardiorespiratory recordings were performed on 17 infants. PGE_2 levels in CSF were correlated with apnea, bradycardia and desaturation (ABD) events, ($P < .05$) (Fig. 12, A). ROC curves illustrated greater specificity and sensitivity for the correlation of PGEM to ABD events than for CRP (Fig. 12, B).

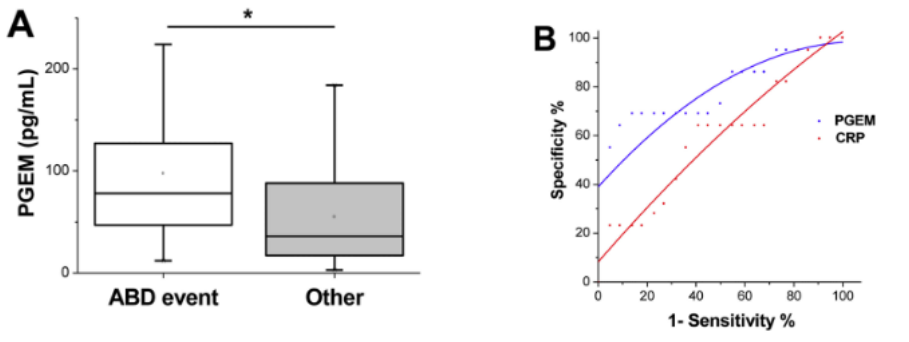

Figure 12. Correlation of PGEM and ABD events (A). Specificity and sensitivity of PGEM correlation with ABD in comparison with CRP (B).

Key points in paper II:

- PGEM levels in CSF are increased in preterm infants compared to term infants
- PGEM levels predicts culture-confirmed sepsis/meningitis with more specificity and sensitivity than CRP
- PGEM correlates with ABD events with higher predictability than CRP

4.3 Study III

Of 64 infants included in this study 44 patients fulfilled the criteria of perinatal asphyxia and 20 were control infants without asphyxia. According to Sarnat et Sarnat clinical evaluation (Sarnat & Sarnat, 1976), 14 of the patients had HIE-I (31,8%), 16 HIE-II (36,4%), and 14 HIE-III (31,8%). Eight infants died in the neonatal period, 16 had adverse neurological outcomes at 18 months and 20 had a normal outcome.

4.3.1 Kinetics of FasL and IL-6 levels in HIE

From 12 of the patients, two samples were obtained. The first at 14 h and the second at 72 h after birth. Tends were observed in FasL and IL-6 levels where FasL rose but IL-6 fell, over time (Fig. 13, A and B).

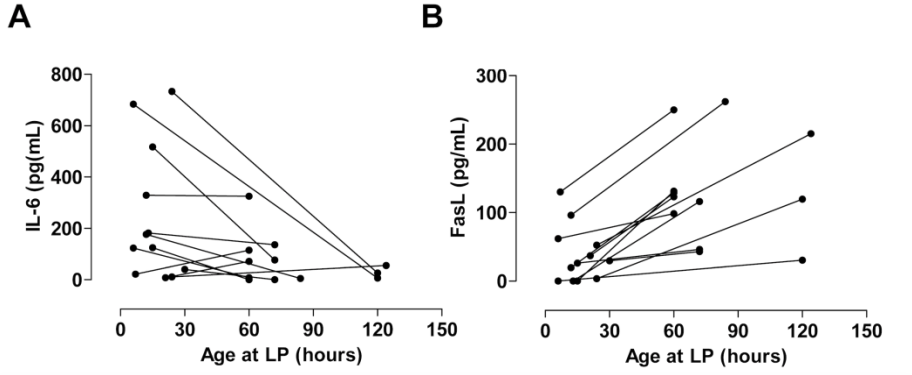

Figure 13. Trends of IL-6 and FasL levels with time.

4.3.2 FasL and IL-6 levels in CSF are indicative of HIE grade

Correlation analysis confirmed that a positive correlation existed between both FasL and IL-6 levels in CSF and the degree of HIE (r=0.6898 and 0.6864, respectively) (p<0.0001) (Fig. 14 A and B).

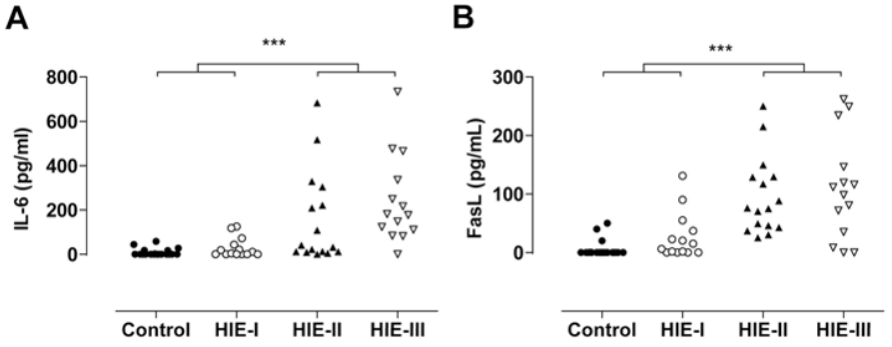

Figure 14. IL-6 and FasL levels correlate with HIE. Data presented as medians and interquartile ranges (IQR). ***p<0.0001

ROC curve was generated of the FasL and IL-6 relation to HIE grade, which gave AUC values of 0.890 and 0.870 respectively (p<0.0001 for both). FasL predicted HIE severity with 90% sensitivity and IL-6 with over 90% specificity. Taken together the AUC was 0.943 and they predicted HIE severity with 86.7% sensitivity and 91.2% specificity (Fig. 15. A, B, and C).

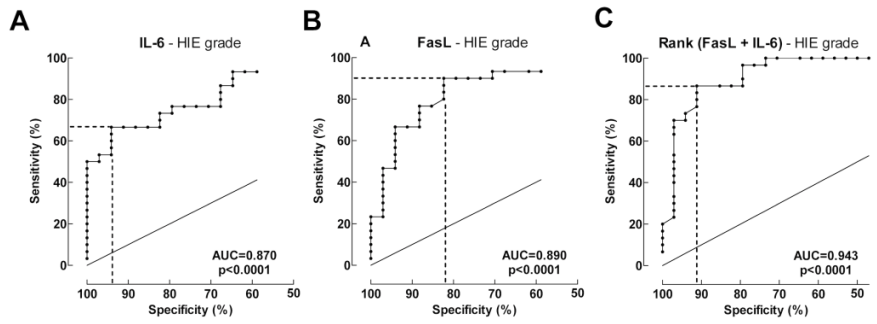

Figure 15. ROC curves of IL-6 and FasL in relation to HIE grade.

4.3.3 FasL and IL-6 levels in CSF predict the outcome

Increased FasL and IL-6 levels correlated with adverse outcomes of patients (r=0.7017 for both) (Fig. 16, A and B). The adverse outcome was defined as death or adverse neurological outcome at 18 months assessment. The neurological outcome was defined as adverse in the case of MDI <85 on BSID assessment, deafness, blindness, cerebral palsy, or epilepsy at 18 months.

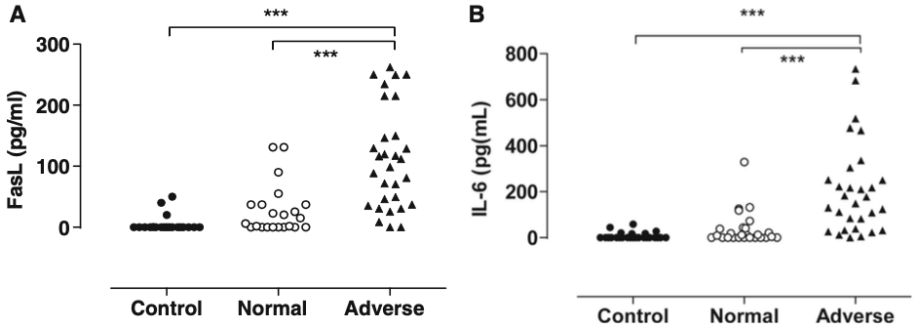

Figure 16. Correlation of FasL and IL-6 with the outcome. Data presented as medians and interquartile ranges (IQR). ***p<0.0001

ROC curves showed that FasL and IL-6 predicted adverse outcomes with 95.8% sensitivity and 85.7% specificity when combined rank for both biomarkers was used (Fig. 17).

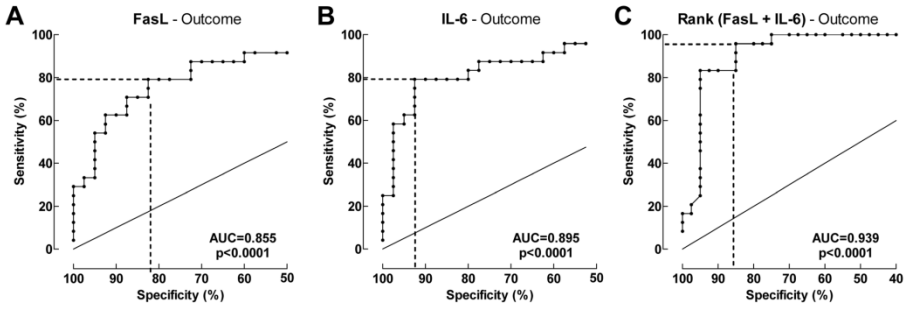

Figure 17. ROC curve of FasL and IL-6 in relation to outcome.

Key points in paper III:

- FasL and IL-6 are found in increased amount in CSF following birth asphyxia in term infants.
- FasL levels rise but IL-6 levels fall in CSF after the insult.
- FasL and IL-6 levels predict HIE severity with high predictability.
- FasL and IL-6 levels predict outcome with high predictability.

4.4 Study IV

Protein profiling in CSF of 18 term infants with HIE (HIE-III, n=7, HIE-II, n=7, HIE-I, n=4) and 10 control infants was conducted utilizing affinity-based antibody suspension bed array technology. From the human protein atlas (HPA), 178 antibodies were chosen for the analysis. Five of the infants died in the neonatal period and n=8 of surviving infants had unfavorable outcomes at 18 months. The controls were infants with suspected infection, but negative cultures in blood and CSF and no brain pathology. They all had a normal outcome.

4.4.1 Protein profile differences

The protein levels were measured as median fluorescent intensities (MFI). Comparison of the relative protein abundances between patients and controls is visualized on a Volcano plot where MFI values are transformed to log_2-changes and plotted against *p*-values (Fig. 18). Several proteins were increased in the CSF of patients compared with controls.

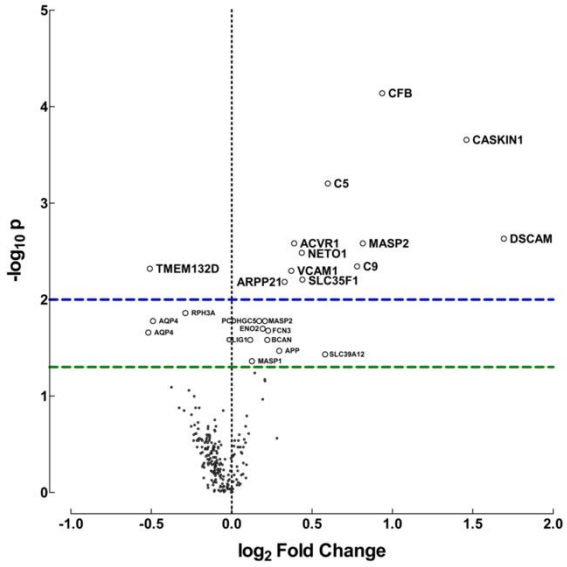

Figure 18. Volcano plot describing data differences between patients and controls.

To explore potential interactions of the highlighted proteins we generated a principal component analysis (PCA) score's plot. Almost identical paths were revealed between the HIE grades and between outcome groups, where a gradient was observed from mild to severe and normal to adverse HIE grades and outcomes, respectively (Fig. 19, A-B).

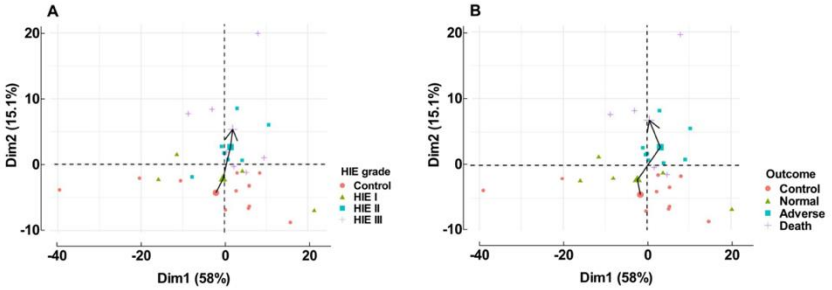

Figure 19. Principal component analysis (PCA) of HIE grade (A) and outcome (B)

Table 4 Protein differences between HIE infants with adverse outcome and controls

Analyte	Protein description	Function	HIE patients with adverse outcome		Control			Log_2 Fold Change	p MWU-test
			Median	IQR	Median	IQR	ΔMFI		
SNCB	Beta-synuclein	4&5	2553	1420–1783	1717	1420–1783	837	0.5727	7.21E–05
ATP6V1G2	V-type proton ATPase subunit G2	2	1194	1022–1338	891	837–915	303	0.4221	7.21E–05
NSE	Neuron-specific enolase	1&2	2231	1900–3764	968	887–1070	1263	1.2046	1.21E–04
ALDOC	Aldolase C	1&2	1578	1275–1951	834	758–984	745	0.9208	1.55E–04
SPTAN1	Spectrin alpha chain	1&3	760	525–1138	414	355–458	346	0.8772	1.98E–04
PRRT2	Proline-rich transmembrane protein 2	5	772	1240–1757	583	832–1299	189	0.4051	1.98E–04
SLC12A5	Solute carrier family 12 member A5	5	1610	1226–2209	957	783–1061	653	0.7501	2.53E–04
RTN1	Reticulon–1	4&5	1480	963–2357	721	649–768	759	1.0370	3.22E–04
SV2A	Synaptic vesicle glycoprotein 2A	5	1029	909–1298	804	705–899	225	0.3555	3.62E–04
ARPP21	cyclic AMP-regulated phosphoprotein 21	2&5	1147	1057–2248	799	686–919	348	0.9408	4.08E–04
DSCAM	Down syndrome cell adhesion molecule	7&8	582	492–867	456	371–474	126	0.5216	4.08E–04
AMER2	APC membrane recruitment protein 2	7	1003	598–2183	523	476–555	481	0.4594	4.08E–04
MBP	Myelin basic protein	1&3	792	728–1004	576	474–606	216	0.3528	4.08E–04
TTC9B	Tetratricopeptide repeat domain 9B	8	732	610–1078	556	507–600	177	0.4483	5.15E–04
NPTX1	Neuronal pentraxin–1	4	2078	1884–2377	1523	1437–1786	555	0.3981	5.15E–04
AQP4	Aquaporin–4	6&7	5592	4707–7303	3747	2663–4056	1845	0.5775	6.47E–04
CNTNAP4	Contactin 4	5&7	718	618–854	525	409–572	193	0.4520	6.47E–04
MOG	Myelin oligodentrocyte glycoprotein	8	1486	1355–1678	1149	1019–1270	337	0.3714	8.11E–04
CASKIN1	CASK-interacting protein 1	5	2153	1864–3528	1023	845–1960	1131	1.0742	1.56E–03
C5	Complement factor 5	7	970	813–1124	686	603–812	284	0.4993	1.56E–03
CFB	Complement factor B	7	2773	2232–3377	1802	1711–2501	971	0.6220	1.93E–03
VCAM1	Vascular adhesion molecule	7	1609	1260–1733	1159	768–1447	450	0.4730	1.93E–03
ACVR1	Activin A receptor type 1	8	1152	1003–1322	873	802–1033	279	0.3995	1.93E–03
RPH3A	Rabphilin 3A	5	693	626–919	548	475–637	145	0.3387	1.93E–03
GAP43	Growth associated protein 43	8	7728	7277–9030	6451	4534–6745	1277	0.2606	2.37E–03
MEPE	Matrix extracellular phophoglycoprotein	3	1074	791–1777	670	548–876	404	0.6808	2.37E–03
HSPA4	Heat shock protein family A member 4	7	551	473–756	411	328–428	141	0.4247	2.63E–03

4.4.2 Pathway analysis of the proteins in CSF

Significant differences (*p*-value <0.05) of several proteins representing different pathological processes in the HI brain injury were observed between the groups. The importance of neuroinflammatory pathways in the HIE infants was confirmed where the complement pathway seemed to be the primary pathway. It discriminated between the severity grades of HIE as well as the outcome of patients (Fig. 20).

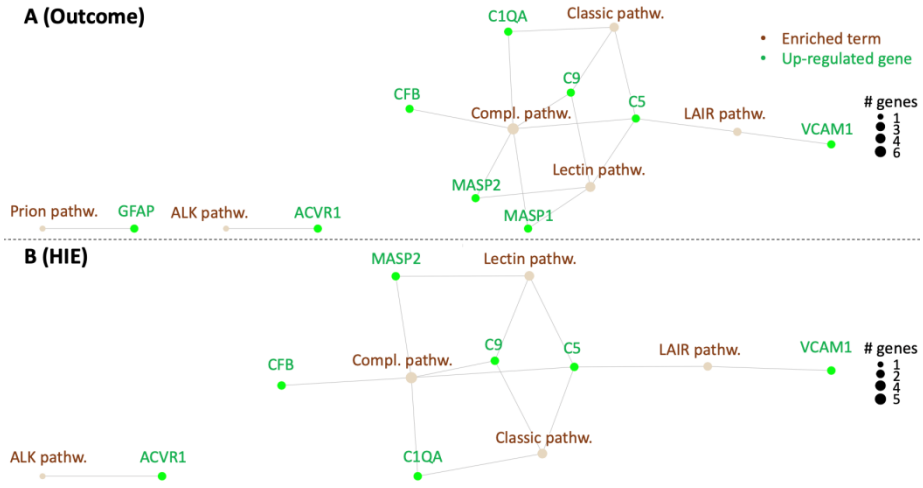

Figure 20. Pathway analysis of protein profiles in correlation with clinical outcome (A) and HIE grade (B).

4.4.3 Correlation with adverse clinical outcomes of patients

For 51 unique CSF proteins, differences were found in relation to outcome (Table 4). The proteins exhibiting the biggest data differences are displayed on a scatter plot (Figure 21).

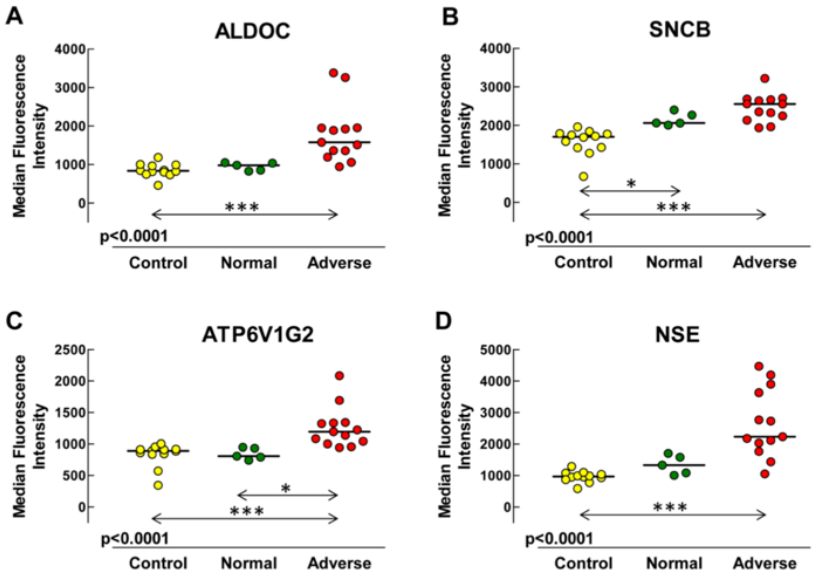

Figure 21. Scatter plot of protein levels in HIE in correlation with outcome

Key points in paper IV:

- Several proteins were increased in CSF of asphyxiated infants and correlated with the degree of HIE and outcome
- Upregulation of inflammatory pathways was observed, with the complement pathway most important in relation to HIE grade and outcome
- 51 proteins that represent different pathological processes of HI brain injury correlated significantly with adverse outcomes of patients

4.5 Study V

In this study comprehensive profiling of protein expression in CSF from 27 preterm infants with gestational age (GA) 26w+4d and 10 full-term control infants was performed utilizing affinity-based antibody suspension bed array technology.

Eleven of the preterm infants had adverse neurological outcomes at 24 months follow-up, but none of the term infants. The definition of adverse neurodevelopmental outcome was identical for the preterm and term infants; CP, seizure disorder, motor delay, mental developmental index <85, deafness or blindness at 18 to 24-month assessment.

4.5.1 Differences in CSF protein levels

Several unique inflammatory proteins and brain-specific proteins reflecting ongoing neurodevelopmental processes were increased in CSF of preterm compared with term infants as displayed on a Volcano plot in Figure 22.

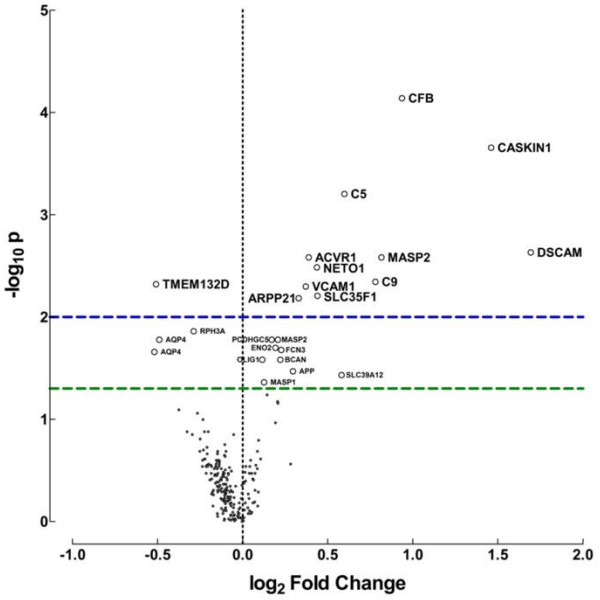

Figure 22. Volcano plot describing data differences between preterm and term infants.

When term and preterm infants with the favorable outcome only were compared, similar protein alterations as described above were observed. (see Table 5)

Table 5. Protein differences between preterm infants and term infants.

			Preterm		Term				
Analyte	**Description**	**Function**	**Median**	**IQR**	**Median**	**IQR**	**ΔMFI**	**log_2 Fold Change**	***p****
CFB	Complement factor B	Neuroinflammation, regulation of immune reaction	3448	3222-4678	1802	1643-2209	**1646**	0.94	7.21E-05
C5	Complement 5	Neurodevelopment and Neuroinflammation	1143	961-1249	686	588-751	**438**	0.74	1.98E-04
CASKIN1	CASK-interacting protein 1	Synaptic plasticity	3753	2294-5863	1022	790-1521	**2731**	1.88	1.98E-04
VCAM1	Vascular cell adhesion molecule 1	Neuroinflammation and early neural proliferation	1672	1478-2002	1159	1105-1411	**513**	0.53	5.15E-04
ARPP21	Cyclic AMP-regulated phosphoprotein 21	Synaptic plasticity	1109	970-1203	799	701-905	**310**	0.47	7.25E-04
MASP2	Mannose-binding protein-associated serine protease 2	Neural migration and Neuroinflammation	1730	1353-3899	982	824-1168	**748**	0.82	8.11E-04
ACVR1	Activin receptor type-1	Neurotrophic & bone morphogenic properties	1165	1075-1431	873	770-953	**292**	0.42	1.26E-03
BCAN	Brevican	Brain developmental & plasticity, maintenance of neural circuitry	1863	1581-1977	1424	1164-1636	**439**	0.39	1.93E-03
NETO1	Neurophilin and tolloid like proten 1	Synaptic plasticity	993	829-1477	712	658-865	**281**	0.48	1.93E-03
DSCAM	Down syndrome cell adhesion molecule	Brain development and Neurotrophic properties	8685	4537-11743	2158	938-4267	**6527**	2.01	1.93E-03
NSE	Neuron specific enolase	Neurotrophic & Neuroprotionproperties	1222	1035-1502	968	892-1085	**254**	0.34	2.37E-03
APP	Amyloid beta precursor protein	Synaptogenesis & Synaptic plasticity	4460	3899-4970	3264	2834-3942	**1196**	0.45	4.33E-03

Proteins in cerebrospinal fluid that exhibited differences in median fluorescent intensity levels at the threshold of $p<0.005$ between term infants and preterm infants with outcome, established by Mann- Whitney U test. Abbreviations; ΔMFI = median fluorescent intensity differences, IQR = inter quartile range. *p = <0.005.

4.5.2 Clinical correlation of CSF protein levels

Several proteins were correlated with the outcome in preterm infants. Most of these proteins are essential for brain function and development. Lower protein levels were observed in preterm infants with adverse outcome than in preterm infants with normal outcomes. Four of the proteins that displayed the largest data differences are visualized on a scatter plot in Figure 23.

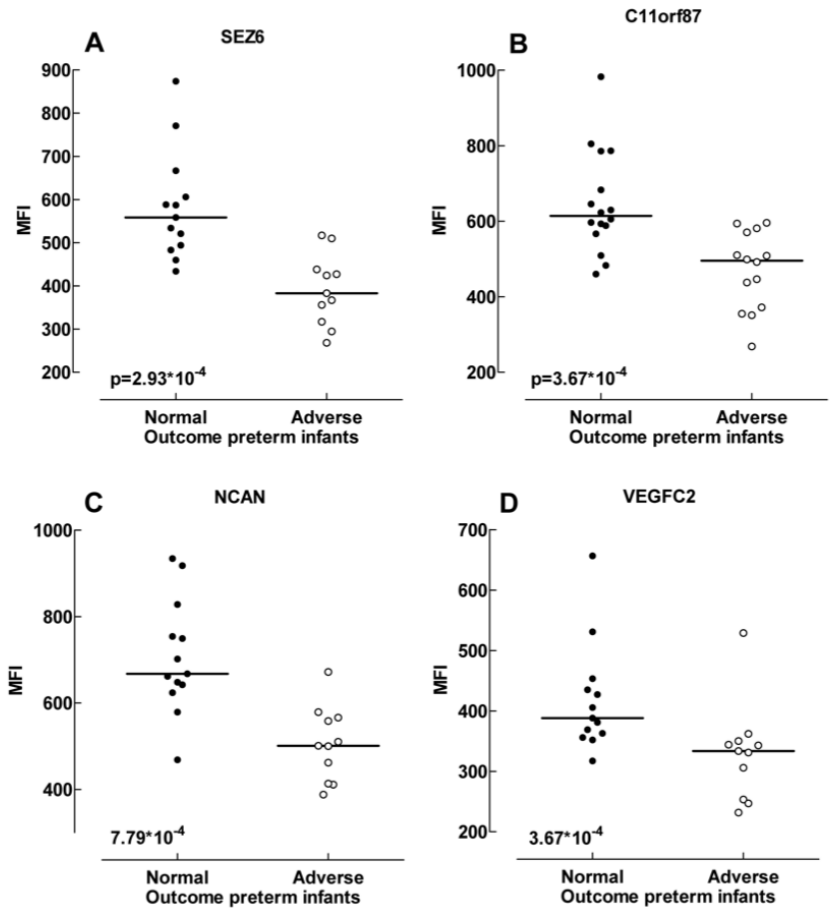

Figure 23. Scatter plot of protein levels in preterm infants in correlation with outcome

Key points in paper V:

- A distinct CSF protein profile was observed in preterm infants compared with term infants.
- Depressed levels of proteins that mediate neurodevelopment correlated with adverse outcome in preterm infants.

5 Discussion

The pathophysiology of brain injury is reflected in CSF better than in other body fluids. In our studies a variety of biochemical substances were measured in the CSF of term and preterm infants that may lead towards a better understanding of the mechanisms of perinatal brain injury. Our results indicate that several of these substances can be considered promising biomarkers of HIE, neonatal sepsis, and preterm neurodevelopmental adversities, having diagnostic and predictive values. It is important to have in mind that the timing of the CSF sampling was later than 6 hours from birth in most infants, which is after the initiation of hypothermia according to todays recommendations for neuroprotective therapy in HIE. None of the infants received therapeutic hypothermia as the recruitment of the patients was in an era prior to the widespread introduction of hypothermia application. The measured biomarkers may therefore not serve as accurate early assessment tools, even though they correlate with the degree of HIE. These proteins may enhance our knowledge of the pathophysiology, reflect the ongoing pathophysiology and lead to novel therapeutic options. Many of these biomarkers have not been investigated previously in infants.

5.1 Hypoxic-ischemic encephalopathy (HIE)

The pathological process leading to HI brain injury of perinatal origin is complex. It includes oxidative stress, apoptosis, breakdown of the BBB, inflammation, and eventually neuronal damage. Therefore, biomarkers identifying neonatal brain damage can have different origins and functions.

In *studies, I, III, and IV* markers that reflect these pathological cascades were measured. They include markers of the main pathological cascades like i) inflammatory biomarkers, ii) markers of apoptosis, and iii) excitatory biomarkers. Also, biomarkers related to the energy exhaustion of cells and structural proteins related to cell breakdown as well as markers of regeneration and neuroplasticity.

The CSF was gathered at a median time of 22.5 (IQR 15–42) hours for the asphyxia group. The measured levels are only a snapshot of an ongoing process at the exact time when the samples were gathered. We do not know the trend of the biomarkers except for PGE_2 in 4 patients and Fas-ligand and IL-6 in 12 patients in study I and III, respectively, who had two LPs taken at two different timepoints.

5.1.1 Inflammatory mediators

An upregulation of the innate immune system, with increased levels of cytokines, like IL-6, IL-16, IL-8, TNF-a, IL1-b and IL-10, in the first days after HI brain injury has been observed in many studies (Massaro et al., 2018). The importance of inflammatory mediators in hypoxia-ischemia has been confirmed in both animal and human studies (Chalak et al., 2014). This is also confirmed in the studies of this thesis. It has been suggested that inflammatory mediators may either be neurotoxic or neuroprotective, depending on the phase in the pathological process of HI brain injury, and the insult severity (Suzuki, Tanaka, & Suzuki, 2009). Infammation has also been correlated with the delayed tertiary phase of the brain injury, which gives hope to a more prolonged therapeutic time window (Hagberg, David Edwards, & Groenendaal, 2016).

Prostaglandins (PGs) are lipid compounds, eicosanoids, exerting hormone-like functions in almost all organs, including the brain. Prostaglandin E_2 (PGE_2) is an important inflammatory mediator and contributes to the pathophysiology of brain ischemia in the developing brain (Ek et al., 2001; Kawano et al., 2006). One of our aims was to evaluate if PGE_2 in CSF would serve as diagnostic marker for the degree of HIE and prognostic marker for the clinical outcome after birth asphyxia. The observed release of PGE_2 into CSF in response to hypoxic insult in term infants in *study I* of this thesis is in line with previous reports on hypoxia mouse models (A. O. Hofstetter et al., 2007; Siljehav, Olsson Hofstetter, Jakobsson, & Herlenius, 2012). Those previous studies illustrate how PGE_2 synthesis in the mouse brain is triggered by anoxia. Further, this process is accelerated by the known inflammatory mediator, IL1β (A. O. Hofstetter et al., 2007). PGE_2 production may also be triggered by other inflammatory mediators, including the cytokine IL-6 (Rummel et al., 2011). In *study III* an association was observed between the CSF levels of prostaglandin E_2 metabolite (PGEM) and IL-6 in CSF in 25 term infants with HIE.

The PGEM, which is a stable metabolite of PGE_2, was measured along with the non-metabolite form of PGE_2. In its original bioactive form, PGE_2 has a very short half-life (Vilanova et al., 1998). The use of PGEM is feasible in clinical settings as it enables a wider observation time and gives information on less transient values. Our results suggest that PGEM can serve as a biomarker for predicting HIE severity and outcome in perinatal asphyxia. This was not demonstrated in a previous clinical study when the level of the non-metabolite form of PGE_2 was analyzed in CSF of asphyxiated infants

(Sumanovic-Glamuzina et al., 2008). Our results demonstrate the importance of analyzing the more stable metabolite of the compound to avoid the rapid turnover of PGE_2 into its metabolites.

The PGEM levels were evaluated in relation to pH and BE, which are well-established indicators of cellular energy failure and used as criteria for defining the intrapartum hypoxia (Practice, 2006). Our results suggest a higher predictive value in relation to the outcome than both pH and BE. This was confirmed by creating ROC curves. This strengthens our results and suggests that PGE_2/PGEM may be valuable biomarkers in HIE.

Interleukin 6 (IL-6) is a cytokine that can either induce or reduce HI brain injury (Suzuki et al., 2009). One of our aims was to study the relationship between IL-6 levels in CSF and the HIE degree as well as the outcome. In *Study III* the data suggest that IL-6 may serve as a biomarker of early HI brain damage and that it has a value in predicting the outcome. Our results described a time-dependent trend of decline in IL-6 levels in HIE patients who had LP at 2 different time points. The observation is in line with earlier reports on IL-6 levels in infants treated under normothermic conditions (Roka et al., 2013).

Orrock et al., 2016 described that IL-6 remained significantly elevated at 72 hours, despsite hypothermia treatment, in infants with adverse outcome. This might indicate infants in need of additional neuroprotective interventions (Orrock et al., 2016) and a neurotoxic function of IL-6 in asphyxia. On the other hand, a neuroprotective role has also been observed in experimental studies where excitotoxicity in the brain is diminished by IL-6 administration following HI injury (X. X. Fang, Jiang, Han, Peng, & Qiu, 2013). A recent study using a Luminex assay showed that raised IL-6 levels in umbilical cord blood differentiated the grades of HIE but did not correlate with neurodevelopmental outcome at 3 years (Ahearne et al., 2017).

Inflammatory pathways of CSF following birth asphyxia were analyzed in *study IV*. We aimed at evaluating the use of antibody microarray, an emerging method in HIE research, and identify the proteome that correlated with the severity of the brain injury and the clinical outcome. Several immune-related proteins were increased in asphyxiated infants compared with controls and correlated with adverse outcomes. Lectin and the complement pathways were the most important pathways in relation to both HIE grade and outcome. Our results confirm the accumulating data that emphasize the value of inflammatory pathways in HI brain injury (B. Li et al., 2017). Inflammatory mediators may be produced in response to the HI insult and

participate in initiating the pathogenesis of the brain injury. Further**,** they may continue to expand for a long time and contribute to the more chronic and prolonged phase in HIE (Hagberg et al., 2015). Therefore the recognition of inflammatory reaction in HI brain injury is of uttermost importance as it may become one of the targets of neuroprotective interventions

5.1.2 Apoptotic mediators

Fas-ligand is a key mediator of neuronal apoptosis, which contributes to the majority of nerve cell death in the HI brain injury (Broughton et al., 2009). FasL levels in CSF of asphyxia patients in *study III* correlated with HIE degree and the outcome of the patients, which met one of our aims. It is in line with previous studies indicating neurotoxic properties of FasL in asphyxia models (Graham et al., 2004). Interestingly though, a number of patients with HIE grade III had low FasL levels in that study. A number of these patients either died or were very sick. Five of the eight patients who died in the cohort had very low Fas-ligand.

Other proteins with apoptotic properties were observed in proteomic analysis of CSF of HIE patients in *study IV*. These included beta synuclein (SNCB), reticulon-1 (RTN1), and neuronal pentraxin-1 (NPTX1). There are several reports on the importance of these proteins in the pathophysiology of neurological diseases (Gong et al., 2017; Sargent et al., 2018) and hypoxia brain injury models (Thatipamula & Hossain, 2014). These mediators have not been investigated previously in the developing brain.

5.1.3 Markers of axonal and cellular damage

Cell structural proteins are released only upon cell damage. Several proteins were significantly increased in HIE infants in *study IV* and correlated with outcome, including myelin basic protein (MBP), alpha II-spectrin (SPTAN), and matrix extracellular phosphoglycoprotein (MEPE). These proteins have been correlated with ischemic and traumatic brain injury (TBI) in experimental and clinical studies before (Beers et al., 2007; Ottens et al., 2010). Our data is in line with previous studies on pediatric TBI suggesting that these proteins may be promising biomarkers of the brain injury (Berger, Hayes, Richichi, Beers, & Wang, 2012). Only few studies are available on the same cell structural proteins that we observed in our HIE cohort (Garcia-Alix et al., 1994; Jain et al., 2014).

The astrocytic skeletal protein glial fibrillary acidic protein (GFAP) is a promising biomarkers for outcome prediction in HIE (Blennow et al., 1995; Chalak et al., 2014). It was increased in CSF in HIE comopared with controls

in our study and the levels were higher in infants with adverse outcome, although the differences were not significant. Tau is another cytoskeletal protein, a known biomarker in Alzheimer's and Parkinson's disease and raised levels have been implicated in HIE (Takahashi et al., 2014). Tau was not measured in our cohorts.

5.1.4 Proteins of brain cell metabolism

Proteins related to cell metabolism may also be markers of cellular damage or exhaustion. These are proteins like neuron specific enolase (NSE), aldolase C (ALDOC), and Adenosine Triphosphatease V-type subunit G2 (ATP6V1G2), which were all highly increased in CSF of HIE patients in *study IV* and correlated with adverse outcomes. NSE and ATP6V1G2 are found in neurons and ALDOC in astrocytes. They are released from the cells after brain cell death. Increased NSE levels in serum and CSF have previously been correlated with brain damage and poor prognosis in HIE (Blennow, Savman, Ilves, Thoresen, & Rosengren, 2001; Kelen et al., 2017). It is a useful biomarker for TBI in adults (Thelin et al., 2016). ALDOC has also been suggested as a promising biomarker in TBI (Halford et al., 2017; Thelin et al., 2018) and ATP6V1G2 correlated with more chronic and progressive brain injuries (Harper et al., 2020). Our results along with the ones of previous studies suggest a clinical utility of these biomarkers in HIE but there is a need for validation in additonal cohorts.

5.1.5 Synaptic proteins

Synaptic regulatory proteins are not well studied in HIE but they take part in exitotoxic neurotransmitter release and can induce cell damage in HI brain injury (Gong et al., 2017). The synaptic proteins may also be indicative of neuroplasticity.

Proteins of synaptic regulation and synaptic plasticity were found in increased amounts in the CSF proteomic analysis of asphyxia patients in study IV. This could be explained by seizures, that are common in infants with HIE-II, but this was not evaluated. Also, it could represent a high turnover of the proteins in relation to the synaptic neuroplasticity in the developing brain. Synaptic vesicle glycoprotein 2A (SV2A) was one of these proteins. It is believed to be neuroprotective and has been correlated with seizure control (Ohno & Tokudome, 2017). Several other synaptic proteins were increased and correlated with adverse outcomes of patients in our study. These included RTN1 which regulates neurotransmitter release and may exaggerate HI brain injury through excitotoxicity, as is suggested in

experimental studies (Gong et al., 2017). Also, SNCB is a synaptic protein with apoptotic potentials found in several neurological diseases (Sargent et al., 2018). The relation of these proteins to HIE has not been investigated previously. Nevertheless, agents reducing excitotoxicity and thereby possibly the expression of the synaptic proteins are being investigated as promising neuroprotective therapies (Filippi et al., 2018).

5.1.6 How cooling may modulate the observed HIE biomarkers. Expansion into clinical use.

The evolution of inflammatory mediators over time may be altered by hypothermia as evidenced by studies where cooled and noncooled cohorts are compared. In a randomised trial, Jenkins et al., 2012, described higher IL-6, IL-8, IL-10 serum levels in cooled infants compared with noncooled. Elevated IL-6 levels in the first 9 hours correlated with death and severe neurodevelopmental outcome at 12 months. Interestingly a bipasic elevation of IL-6 levels was observed at around 24h in the cooled group. A uniform downmodulation down to a trend reversal at 36h in that group correlated with good outcome (Jenkins et al., 2012). This might indicate that the delayed elevation of IL-6 in the cooled group may be a marker of activated repair mechanisms and that the hypothermia might shorten the time to initiate this recovery. In *study III* of this thesis two samples were available at different timepoints from 12 infants. Lower IL-6 levels were observed in the later sample from 9 infants, all who had adverse outcome, but a slight increase was observed in 3 samples and two of them had normal outcomes. This may indicate a different function of IL-6 in noncooled infants than in those who received hypothermia. The time at the late LP was between 60 and 144 hours, but the early LP between 6 and 28 hours. Thus, the early sample is closer to the observed secondary peak in Jenkins et al.

Another clinical study comparing cooled and noncooled HIE infants showed increased IL-6 and IL-10 in the noncooled group. This was a very small cohort and no outcome data (Moon, Youn, Yum, & Sung, 2016). Furthermore, elevated levels of nucleated red blood cells (NRBC) in serum in the first 24 hours, discriminated between mild HIE and moderate to severe HIE in normothermic infants but not in infants who received hypothermia (Walsh, Boylan, Dempsey, & Murray, 2013). This was due to a decrease in NRBCs among moderately encephalopathic cooled infants. Increased NRBC count has been correlated with adverse outcome in perinatal asphyxia (Walsh, Boylan, & Murray, 2011). The rapid elevation immediately after the

hypoxic insult is thought to be secondary to raised IL-6 (Ferber, Minior, Bornstein, & Divon, 2005).

In a piglet HI injury study in cooled and noncooled animals a pro-inflammatory cytokine surge was observed after rewarming in the cooled group (Rocha-Ferreira et al., 2017). This has not been confirmed in clinical studies. Chalak et al described in a small cohort that cytokine values were unaffected by hypothermia-rewarming (Chalak et al., 2014).

These findings, indicating the role of therapeutic hypothermia in immune modulation suggests that neuroinflammation contributes directly to the HI brain injury. Thus the immune mediators are not merely markers of the brain injury but also participants in the pathology. This opens the possibility of further immune modulating neuroprotective therapies.

Brain specific proteins may also be modulated by therapeutic hypothermia. This was indicated in a recent study on 178 infants with HIE who all received therapeutic hypothermia. After hypothermia treatment increased levels of tau and GFAP were observed, which may reflect the role of hypothermia in delaying activation of injurious pathways (Chavez-Valdez et al., 2021). Another study indicated the influence of hypothermia on S-100B and Enolase in serum in a small group of infants. In that study the biomarkers were highly elevated following asphyxia and associated with outcome but the levels were lower in infants treated with hypothermia compared with those who were not (Roka et al., 2012).

Experimental studies have suggested that hypothermia may ameliorate the Fas-mediated apoptotic pathways (X. Liu et al., 2013) and reduced soluble FasL levels were observed in hypothermic brains of experimental stroke models (L. Liu et al., 2008). In our cohort the levels of FasL were higher at later timeponts in the 12 patients who had two samples. This indicates ongoing apoptotic cell death. FasL levels could provide important information about ongoing apoptotic brain damage during and after hypothermia treatment and thus the need for complementary treatment. It could also give information on treatment efficacy. As none of the infants in our cohort received hypothermia these data need to be validated in an additional cohort of cooled infants.

5.2 Neonatal sepsis

Invasive neonatal infections carry a high risk of mortality and neurological morbidities, especially among preterm infants (Weston et al., 2011). Altered neurodevelopment is a complication of neonatal sepsis (Schlapbach et al., 2011). Sepsis presents with apnea, bradycardia, and desaturation (ABD) as

core symptoms (Sullivan, Grice, Lake, Moorman, & Fairchild, 2014). Therefore, we wanted to evaluate the relationship between PGE_2 and apnea. Apnea of prematurity is considered a risk factor for adverse neurodevelopment, even without an association with infections (Janvier et al., 2004; Yang et al., 2021). It is therefore essential to comprehend the mechanisms of ABD events in correlation to infections. Besides its role as an inflammatory mediator, PGE_2 is involved in fetal and neonatal respiratory control by affecting the respiratory rhythm generating neurons in the brain stem (Herlenius, 2011). In *study II* the role of PGE_2 in mediating apnea and cardiorespiratory dysregulation was confirmed, as has been shown previously in an experimental study (Hofstetter, Legnevall, Herlenius, & Katz-Salamon, 2008). Our results provide information about the involvement of prostaglandins in the mechanisms underlying autonomic dysfunction in correlation with infections. ROC curves illustrated a correlation between PGE_2/PGEM levels and ABD events with more sensitivity and specificity than the correlation between CRP and ABD events. Furthermore, our results confirm that PGE_2 levels are higher in preterm infants compared with term infants in the first days of life (Agostiniani et al., 2002). This observation possibly indicates a relationship between preterm-specific inflammation and apneas.

The initial signs of sepsis in the neonatal period are often subtle and nonspecific, which makes the early diagnosis difficult. PGE_2 and PGEM levels in *study II* demonstrated a better correlation between culture-verified bacterial infections and PGE_2 than CRP. This was illustrated with ROC curves**,** showing more sensitivity and precision. We, therefore, suggest that PGE_2 and PGEM may be useful biomarkers in the early diagnosis of sepsis and patient surveillance, especially in cases where the infection is suspected but the initial signs and symptoms are nonspecific. Furthermore, we suggest that analyzing PGEM levels in combination with monitoring ABD events could increase the accuracy of early diagnosis of infection. This needs further validation in a bigger cohort.

5.3 Outcome prediction in preterm infants

As discussed in the previous section, PGE_2 levels were higher in CSF of preterm infants than in term infants in *study II*. Biochemical biomarkers in CSF of preterm infants analyzed in *study V* are discussed below.

5.3.1 CSF proteome reflecting neuroinflammation

The goal of *study V* was to compare the proteome of preterm infants and term-born infants. Also, to identify a proteome that would predict the neurodevelopmental outcome in preterm infants. In line with previous studies in preterm infants, we observed an upregulation of inflammatory proteins in preterm compared with term infants (Boardman et al., 2018; Pataky, Howie, Girardi, & Boardman, 2017). There is evidence indicating that upregulation of neuroinflammation is associated with adverse neurodevelopmental outcomes in preterm infants (Allred et al., 2017). In our study, the data differences were not explained by blood or CSF culture verification of sepsis or meningitis. Furthermore, no differences in CRP levels were found between the preterm and term infants. Proteins associated with the complement cascade were prominent. Although neuroinflammation is associated with brain injury, opposing roles of inflammatory mediators have been observed. Notably, there is evidence from previous studies that various components of the complement system have an important role as mediators of brain development and neuroplasticity (Biggins et al., 2017; Magdalon et al., 2020). Preterm brain injury is often primarily the result of disturbed neurodevelopment, sometimes without apparent brain injury. TBI and HIE are associated with brain tissue defects and the formation of scar tissue although it may lead to neurodevelopmental disturbance as well. This may be an explanation of the different functions of the inflammatory mediators.

The importance of the interrelated functions of the immune- and nervous systems in the developing brain is underlined in *study V*.

5.3.2 Markers of neurodevelopmental outcome

Higher CSF levels of several neurodevelopmentally associated proteins were observed in the preterm group compared with the term infants in *study V*. This could reflect the rapid developmental processes of the preterm brain. Notably, lower protein levels correlated with the adverse neurodevelopmental outcome of the preterm infants, which is in line wiht previous studies. Kuban et al., 2018 demonstated that higher levels of neurotrophic proteins correlated with better cognitive functions (Kuban et al., 2018). Decreased levels of several mediators of neurodevelopment in serum of preterm infants have been found to correlate with retinopathy of prematurity (ROP), which is a disease that is mostly seen in the lowest gestational ages (Danielsson et al., 2021).

Among the proteins displaying the biggest data differences between outcome groups in our study was VEGFC**.** This is a protein that has been

shown to induce the growth and differentiation of neuro-progenitor cells as well as regulate angiogenesis in the brain (Le Bras et al., 2006). Recently the administration of VEGF improved neurological outcomes after TBI in rat model (Ju et al., 2019).

Depressed levels of several proteins of neuronal migration and plasticity were observed in correlation with adverse outcomes in *study V*. These included the proteoglycan Neurocan core protein (NCAN) and seizure protein 6 (Sez6). NCAN is important for critical steps in the cognitive and behavioral development (Schwartz & Domowicz, 2018). The lack of Sez6 has been correlated with motor impairment and memory deficits (Nash et al., 2020). Also, we found lower levels of neuron-specific T-box transcription factor 1 (TBR1), in relation to adverse outcomes. This protein has recently been discovered with gene sequencing to have an important role in cortical development and cognitive function (Vegas et al., 2018). In a preclinical study on rodents, mutations in the TBR1 gene resulted in autistic spectrum disease (ASD) like behavior (Yook et al., 2019). ASD is a neurodevelopmental disorder that is seen at much higher rates in preterm infants than in infants born at term age (Agrawal et al., 2018). Preterm infants also have a greatly increased risk of neuropsychiatric disorders, including schizophrenia (Vanes et al., 2021). Like in preterm infants, altered synaptic plasticity and behavioral pathways in the brain have been associated with schizophrenia (Fromer et al., 2014). In our study, we found lower CSF levels in the adverse outcome group of chromosomes 11 open reading frame 87 (c11orf87) protein, also known as neuronal integral membrane protein 1. This protein has been correlated with schizophrenia-related pathways (Etemadikhah, Niazi, Wetterberg, & Feuk, 2020).

5.4 Summary of discussion

Our findings of the five studies confirmed our hypothesis that brain-specific and immune related biomarkers in CSF can predict brain injury and the prognosis of preterm and term infants. Our aims were met. Furthermore, the results add to the knowledge of the pathophysiolgy of HI brain injury and dysmaturation in the developing brain. This is important as better understanding of the mechanisms of injury may eventually lead to novel therapeutic options. CSF does have the advantage of being a better reflection of brain physiology than other body fluids. Our results may be indicative of possibilities for novel therapeutic interventions in both term and preterm infants. Also they could help evaluate the extent of brain injuiry and monitor the efficacy of neuroprotective intervention.

The observed biomarkers were predictive of outcome in our cohorts. It is important though to acknowledge that none of the HIE patients received hypothermia. A further research is warranted in a cohort of cooled infants to validate the predictive power of the biomarkers.

5.5 Strengths and Limitations

In these studies, we collected a distinctive CSF biobank from newborn infants. We discovered an array of unprecedented proteins in these samples. Analyzing CSF as opposed to blood samples is a strength as CSF is near the brain and therefore better reflects the biochemical changes in brain injury. Also, for the CSF analysis in *studies IV and V,* we utilized an emerging technique in brain research that allows us to simultaneously quantify large numbers of proteins in small samples. This is highly important for the investigations of protein profiles in infants where sample sizes are limited. Also, it detects simultaneously many proteins, representing different pathological cascades. This is important as it reflects the complex interaction of simultaneous processes that ultimately lead to the injury.

Several limitations are acknowledged. There was a long time interval between patient recruitment and the sample analysis, which could affect the outcome as some proteins might deteriorate in the freezer over time. The limited number of recruited patients is a limitation and calls for further investigations in larger cohorts. The controls were not truly healthy controls as they were subjected to LP on clinical indications. It is possible that some may have had culture negative sepsis.

None of the infants in the cohort received therapeutic hypothermia as it was not a recommended treatment for asphyxia at the time of recruitment. This is a limitation, especially when comparing the results with recent studies. On the other hand, it could also give important information on the brain HI injury pathology, without the impact of TH. Another limitation is the clinical follow-up, which was not done as a part of the study protocol but rather as a part of a clinical rutine. It was performed by senior neurologists, neonatologists, and physiotherapists, and the information was gathered from the infants' journals. Bayley developmental evaluation was performed only on those who were suspected to have abnormal neurodevelopment at 18-24 months, but not on those without any clinical signs or suspicion. In the proteomic studies (studies IV and V), the protein selection for the antibody array was based on previous studies, which is unavoidably biased.

6 Conclusions

The main purpose of this thesis was to uncover reliable biomarkers in cerebrospinal fluid for precise and early assessment as well as outcome prediction of infants at risk of adverse outcomes following preterm birth, hypoxic brain insults, or sepsis. Several promising results have been revealed in previous studies but currently, no specific biomarker of perinatal brain injury is available for clinical use, and it is an ongoing work.

The overall aims were met as we observed several biomarkers of diagnostic and predictive value reflecting diverse origin and function of the pathological processes leading to perinatal brain injury.

6.1 Neuroinflammation

Inflammatory mediators like the cytokine IL-6 and the eicosanoid PGE_2 had diagnostic values for the severity of HIE and sepsis. These mediators correlated with the unfavorable outcome of the infants. PGE_2 was also observed in association with apnea, bradycardia, and desaturation (ABD) events. Neuroinflammatory upregulation was further confirmed with an antibody microarray analysis and pathway analysis where a neuroinflammatory protein profile was observed. The complement pathway was most prominent in both term infants with HIE and preterm infants.

There are important implications to these findings. Inflammatory mediators can lead to a prolonged and persistent phase of hypoxic brain injury which can alter normal brain development. The recognition of this important pathological phase in perinatal brain injury opens new possibilities to alternative therapeutic modalities. To aid the repair mechanisms in the brain and promote brain cell regeneration, may be as important as diminishing the injurious process itself. This could be possible even long after the insult. Therapies are being explored that target neuronal regeneration at later stages, after the secondary phase of hypoxic ischemia. This can possibly be achieved with growth factors and stem cell therapies. Neuroinflammation is also a contributor to the pathological processes of brain injury in the immature preterm brain. It has been established that the immune and nervous systems are interrelated and interactive. Brain injury in preterm infants is correlated with sepsis during the neonatal period. It has been suggested that neuroinflammation may alter neurodevelopmental outcomes

by altering gene expression. Notably, evidence indicates that inflammatory proteins can also function as important mediators of brain development. Our results contribute to the understanding of the developing brain and the importance of the interaction between the nervous and immune systems.

6.2 Biomarkers reflecting the injurious processes in HIE

Proteins related to cell metabolism, reflective of the energy state of the nerve cells, and structural proteins indicative of cellular damage and breakdown, were increased in CSF of infants who suffered birth asphyxia. Increased levels of apoptotic mediators like Fas-ligand, indicating activity of the secondary phase of nerve cell death were found. Also, synaptic regulatory proteins that could reflect increased excitability of the nerve cells. Furthermore, proteins with neurotrophic properties, possibly indicating an active regenerative process, were found. A correlation between increased levels of these markers and both more severe HIE and worse outcomes of the infants was observed.

These findings may pave the way to a better understanding of the HI brain injury as these proteins can serve as important indicators of the various processes in the HI brain injury pathology. Thus, they could also provide important information for determining the right timing and duration of adjuvant therapy alongside hypothermia. The goal is to invent neuroprotective strategies to improve outcomes. Targeting common mediators of neuronal cell death in hypoxic brain injury offers a potential approach for developing new neuroprotective strategies. Biomarkers could also serve as indicators of the effectiveness and safety of such therapeutic interventions. No licensed additive pharmacological treatment is currently available, but several studies are ongoing.

6.3 Neurodevelopmental outcomes in preterm infants

CSF levels of proteins that are associated with brain development were lower in preterm infants who had adverse neurodevelopment compared with preterm infants with normal outcome.

Less is known about the pathophysiology and etiology of brain injury in preterm infants than in term-born infants. The contributing factors are likely more heterogeneous. The neurological sequelae result at least in part from the brain's immaturity and vulnerability of the developmental processes. Therefore our results are highly relevant. They provide important information regarding essential proteins for optimal brain development, some of which have not been investigated previously in the developing brain. A proteomic signature is revealed that may reflect changes in neurodevelopment associated with external or internal events. Recent studies show that preterm

infants have a common protein profile at birth that is related to the gestational age and that protein alterations occur after birth, depending on postnatal events.

There is an urgent need for improved methods for the diagnosis of brain injury in preterm infants. The timing, severity, and evolution. The observed downregulation of essential proteins for brain development in our study opens possibilities of targeted and individually directed interventions in preterm infants. It could also help to identify factors that may influence the brain's maturational processes, like nutritional, infectious, and stress-related factors.

Our results in the five studies of this thesis enhance our understanding of neonatal brain injury, in term as well as preterm infants, whether caused by hypoxia, infections, or other external stimuli that may cause brain dysmaturation. A better understanding of the pathological processes will hopefully lead to better diagnosis, and improved care and treatment of perinatal brain injury, rendering improved survival and outcome for these infants.

References

Agostiniani, R., Mariotti, P., Cataldi, L., Fanos, V., Sani, S., Zaccaron, A., & Cuzzolin, L. (2002). Role of renal PGE2 in the adaptation from foetal to extrauterine life in term and preterm infants. Prostaglandins Leukot Essent Fatty Acids, 67(6), 373-377. doi:10.1054/plef.2002.0444

Agrawal, S., Rao, S. C., Bulsara, M. K., & Patole, S. K. (2018). Prevalence of Autism Spectrum Disorder in Preterm Infants: A Meta-analysis. Pediatrics, 142(3). doi:10.1542/peds.2018-0134

Ahearne, C. E., Chang, R. Y., Walsh, B. H., Boylan, G. B., & Murray, D. M. (2017). Cord Blood IL-16 Is Associated with 3-Year Neurodevelopmental Outcomes in Perinatal Asphyxia and Hypoxic-Ischaemic Encephalopathy. Dev Neurosci, 39(1-4), 59-65. doi:10.1159/000471508

Ahmed, J., Pullattayil, S. A., Robertson, N. J., & More, K. (2021). Melatonin for neuroprotection in neonatal encephalopathy: A systematic review & meta-analysis of clinical trials. Eur J Paediatr Neurol, 31, 38-45. doi:10.1016/j.ejpn.2021.02.003

Allred, E. N., Dammann, O., Fichorova, R. N., Hooper, S. R., Hunter, S. J., Joseph, R. M., . . . Investigators, E. S. A. s. w. g. f. t. E. S. (2017). Systemic Inflammation during the First Postnatal Month and the Risk of Attention Deficit Hyperactivity Disorder Characteristics among 10 year-old Children Born Extremely Preterm. J Neuroimmune Pharmacol, 12(3), 531-543. doi:10.1007/s11481-017-9742-9

Aly, H., Elmahdy, H., El-Dib, M., Rowisha, M., Awny, M., El-Gohary, T., . . . El-Mashad, A. R. (2015). Melatonin use for neuroprotection in perinatal asphyxia: a randomized controlled pilot study. J Perinatol, 35(3), 186-191. doi:10.1038/jp.2014.186

Amer, R., Moddemann, D., Seshia, M., Alvaro, R., Synnes, A., Lee, K. S., . . . Canadian Neonatal Follow-up Network, I. (2018). Neurodevelopmental Outcomes of Infants Born at <29 Weeks of Gestation Admitted to Canadian Neonatal Intensive Care Units Based on Location of Birth. J Pediatr, 196, 31-37 e31. doi:10.1016/j.jpeds.2017.11.038

Andreasson, K. (2010). Emerging roles of PGE2 receptors in models of neurological disease. Prostaglandins Other Lipid Mediat, 91(3-4), 104-112. doi:10.1016/j.prostaglandins.2009.04.003

Annink, K. V., de Vries, L. S., Groenendaal, F., Eijsermans, R., Mocking, M., van Schooneveld, M. M. J., . . . van der Aa, N. E. (2021). Mammillary body atrophy and other MRI correlates of school-age outcome following neonatal hypoxic-ischemic encephalopathy. Sci Rep, 11(1), 5017. doi:10.1038/s41598-021-83982-8

Annink, K. V., Franz, A. R., Derks, J. B., Rudiger, M., Bel, F. V., & Benders, M. (2017). Allopurinol: Old Drug, New Indication in Neonates? Curr Pharm Des, 23(38), 5935-5942. doi:10.2174/1381612823666170918123307

Athauda, D., & Foltynie, T. (2018). Protective effects of the GLP-1 mimetic exendin-4 in Parkinson's disease. Neuropharmacology, 136(Pt B), 260-270. doi:10.1016/j.neuropharm.2017.09.023

Austin, T. (2018). Therapeutic hypothermia for hypoxic-ischemic encephalopathy: challenges during transfer and global perspectives. J Pediatr (Rio J), 94(3), 221-223. doi:10.1016/j.jped.2017.09.002

Azzopardi, D., Robertson, N. J., Bainbridge, A., Cady, E., Charles-Edwards, G., Deierl, A., . . . Edwards, A. D. (2016). Moderate hypothermia within 6 h of birth plus inhaled xenon versus moderate hypothermia alone after birth asphyxia (TOBY-Xe): a proof-of-concept, open-label, randomised controlled trial. Lancet Neurol, 15(2), 145-153. doi:10.1016/S1474-4422(15)00347-6

Azzopardi, D., Strohm, B., Marlow, N., Brocklehurst, P., Deierl, A., Eddama, O., . . . Group, T. S. (2014). Effects of hypothermia for perinatal asphyxia on childhood outcomes. N Engl J Med, 371(2), 140-149. doi:10.1056/NEJMoa1315788

Azzopardi, D., Wyatt, J. S., Cady, E. B., Delpy, D. T., Baudin, J., Stewart, A. L., . . . Reynolds, E. O. (1989). Prognosis of newborn infants with hypoxic-ischemic brain injury assessed by phosphorus magnetic resonance spectroscopy. Pediatr Res, 25(5), 445-451. doi:10.1203/00006450-198905000-00004

Back, S. A. (2014). Cerebral white and gray matter injury in newborns: new insights into pathophysiology and management. Clin Perinatol, 41(1), 1-24. doi:10.1016/j.clp.2013.11.001

Back, S. A. (2015). Brain Injury in the Preterm Infant: New Horizons for Pathogenesis and Prevention. Pediatr Neurol, 53(3), 185-192. doi:10.1016/j.pediatrneurol.2015.04.006

Back, S. A. (2017). White matter injury in the preterm infant: pathology and mechanisms. Acta Neuropathol, 134(3), 331-349. doi:10.1007/s00401-017-1718-6

Back, S. A., & Miller, S. P. (2014). Brain injury in premature neonates: A primary cerebral dysmaturation disorder? Ann Neurol, 75(4), 469-486. doi:10.1002/ana.24132

Back, S. A., & Rosenberg, P. A. (2014). Pathophysiology of glia in perinatal white matter injury. Glia, 62(11), 1790-1815. doi:10.1002/glia.22658

Baliga, S. S., Merrill, G. F., Shinohara, M. L., & Denhardt, D. T. (2011). Osteopontin expression during early cerebral ischemia-reperfusion in rats: enhanced expression in the right cortex is suppressed by acetaminophen. PLoS One, 6(1), e14568. doi:10.1371/journal.pone.0014568

Ballanyi, K., Lalley, P. M., Hoch, B., & Richter, D. W. (1997). cAMP-dependent reversal of opioid- and prostaglandin-mediated depression of the isolated respiratory network in newborn rats. J Physiol, 504 (Pt 1), 127-134. doi:10.1111/j.1469-7793.1997.127bf.x

Barnett, M. L., Tusor, N., Ball, G., Chew, A., Falconer, S., Aljabar, P., . . . Counsell, S. J. (2018). Exploring the multiple-hit hypothesis of preterm white matter damage using diffusion MRI. Neuroimage Clin, 17, 596-606. doi:10.1016/j.nicl.2017.11.017

Bartel, D. P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. Cell, 116(2), 281-297. doi:10.1016/s0092-8674(04)00045-5

Beers, S. R., Berger, R. P., & Adelson, P. D. (2007). Neurocognitive outcome and serum biomarkers in inflicted versus non-inflicted traumatic brain injury in young children. J Neurotrauma, 24(1), 97-105. doi:10.1089/neu.2006.0055

Beijer, D., Deconinck, T., De Bleecker, J. L., Dotti, M. T., Malandrini, A., Urtizberea, J. A., . . . Baets, J. (2019). Nonsense mutations in alpha-II spectrin in three families with juvenile onset hereditary motor neuropathy. Brain, 142(9), 2605-2616. doi:10.1093/brain/awz216

Bell, E. F., Hintz, S. R., Hansen, N. I., Bann, C. M., Wyckoff, M. H., DeMauro, S. B., . . . Human Development Neonatal Research, N. (2022). Mortality, In-Hospital Morbidity, Care Practices, and 2-Year Outcomes for Extremely Preterm Infants in the US, 2013-2018. JAMA, 327(3), 248-263. doi:10.1001/jama.2021.23580

Benders, M. J., Bos, A. F., Rademaker, C. M., Rijken, M., Torrance, H. L., Groenendaal, F., & van Bel, F. (2006). Early postnatal allopurinol does not improve short term outcome after severe birth asphyxia. Arch Dis Child Fetal Neonatal Ed, 91(3), F163-165. doi:10.1136/adc.2005.086652

Bennet, L., Roelfsema, V., George, S., Dean, J. M., Emerald, B. S., & Gunn, A. J. (2007). The effect of cerebral hypothermia on white and grey matter injury induced by severe hypoxia in preterm fetal sheep. J Physiol, 578(Pt 2), 491-506. doi:10.1113/jphysiol.2006.119602

Berger, R. P., Beers, S. R., Richichi, R., Wiesman, D., & Adelson, P. D. (2007). Serum biomarker concentrations and outcome after pediatric traumatic brain injury. J Neurotrauma, 24(12), 1793-1801. doi:10.1089/neu.2007.0316

Berger, R. P., Hayes, R. L., Richichi, R., Beers, S. R., & Wang, K. K. (2012). Serum concentrations of ubiquitin C-terminal hydrolase-L1 and alphaII-spectrin breakdown product 145 kDa correlate with outcome after pediatric TBI. J Neurotrauma, 29(1), 162-167. doi:10.1089/neu.2011.1989

Bhalala, U. S., Koehler, R. C., & Kannan, S. (2014). Neuroinflammation and neuroimmune dysregulation after acute hypoxic-ischemic injury of developing brain. Front Pediatr, 2, 144. doi:10.3389/fped.2014.00144

Biggins, P. J. C., Brennan, F. H., Taylor, S. M., Woodruff, T. M., & Ruitenberg, M. J. (2017). The Alternative Receptor for Complement Component 5a, C5aR2, Conveys Neuroprotection in Traumatic Spinal Cord Injury. J Neurotrauma, 34(12), 2075-2085. doi:10.1089/neu.2016.4701

Blaise, B. J., Schwendimann, L., Chhor, V., Degos, V., Hodson, M. P., Dallmann, G., . . . Fleiss, B. (2017). Persistently Altered Metabolic Phenotype following Perinatal Excitotoxic Brain Injury. Dev Neurosci, 39(1-4), 182-191. doi:10.1159/000464131

Blencowe, H., Cousens, S., Oestergaard, M. Z., Chou, D., Moller, A. B., Narwal, R., . . . Lawn, J. E. (2012). National, regional, and worldwide estimates of preterm birth rates in the year 2010 with time trends since 1990 for selected countries: a systematic analysis and implications. Lancet, 379(9832), 2162-2172. doi:10.1016/S0140-6736(12)60820-4

Blennow, M., Hagberg, H., & Rosengren, L. (1995). Glial fibrillary acidic protein in the cerebrospinal fluid: a possible indicator of prognosis in full-term asphyxiated newborn infants? Pediatr Res, 37(3), 260-264. doi:10.1203/00006450-199503000-00002

Blennow, M., Savman, K., Ilves, P., Thoresen, M., & Rosengren, L. (2001). Brain-specific proteins in the cerebrospinal fluid of severely asphyxiated newborn infants. Acta Paediatr, 90(10), 1171-1175. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/11697430

http://onlinelibrary.wiley.com/store/10.1111/j.1651-2227.2001.tb03249.x/asset/j.1651-2227.2001.tb03249.x.pdf?v=1&t=j9spp12c&s=cda85d5a218d1428e10d68faf4cc4a42c5044c14

Boardman, J. P., Craven, C., Valappil, S., Counsell, S. J., Dyet, L. E., Rueckert, D., . . . Edwards, A. D. (2010). A common neonatal image phenotype predicts adverse neurodevelopmental outcome in children born preterm. Neuroimage, 52(2), 409-414. doi:10.1016/j.neuroimage.2010.04.261

Boardman, J. P., Ireland, G., Sullivan, G., Pataky, R., Fleiss, B., Gressens, P., & Miron, V. (2018). The Cerebrospinal Fluid Inflammatory Response to Preterm Birth. Front Physiol, 9, 1299. doi:10.3389/fphys.2018.01299

Broughton, B. R., Reutens, D. C., & Sobey, C. G. (2009). Apoptotic mechanisms after cerebral ischemia. Stroke, 40(5), e331-339. doi:10.1161/STROKEAHA.108.531632

Brywe, K. G., Mallard, C., Gustavsson, M., Hedtjarn, M., Leverin, A. L., Wang, X., . . . Hagberg, H. (2005). IGF-I neuroprotection in the immature brain after hypoxia-ischemia, involvement of Akt and GSK3beta? Eur J Neurosci, 21(6), 1489-1502. doi:10.1111/j.1460-9568.2005.03982.x

Bystron, I., Blakemore, C., & Rakic, P. (2008). Development of the human cerebral cortex: Boulder Committee revisited. Nat Rev Neurosci, 9(2), 110-122. doi:10.1038/nrn2252

Cady, E. B., Iwata, O., Bainbridge, A., Wyatt, J. S., & Robertson, N. J. (2008). Phosphorus magnetic resonance spectroscopy 2 h after perinatal cerebral hypoxia-ischemia prognosticates outcome in the newborn piglet. J Neurochem, 107(4), 1027-1035. doi:10.1111/j.1471-4159.2008.05662.x

Campbell, I. L., Stalder, A. K., Chiang, C. S., Bellinger, R., Heyser, C. J., Steffensen, S., . . . Siggins, G. R. (1997). Transgenic models to assess the pathogenic actions of cytokines in the central nervous system. Mol Psychiatry, 2(2), 125-129. doi:10.1038/sj.mp.4000225

Candelario-Jalil, E., Taheri, S., Yang, Y., Sood, R., Grossetete, M., Estrada, E. Y., . . . Rosenberg, G. A. (2007). Cyclooxygenase inhibition limits blood-brain barrier disruption following intracerebral injection of tumor necrosis factor-alpha in the rat. J Pharmacol Exp Ther, 323(2), 488-498. doi:10.1124/jpet.107.127035

Chakkarapani, A. A., Aly, H., Benders, M., Cotten, C. M., El-Dib, M., Gressens, P., . . . Publications, C. (2021). Therapies for neonatal encephalopathy: Targeting the latent, secondary and tertiary phases of evolving brain injury. Semin Fetal Neonatal Med, 26(5), 101256. doi:10.1016/j.siny.2021.101256

Chalak, L. F., Sanchez, P. J., Adams-Huet, B., Laptook, A. R., Heyne, R. J., & Rosenfeld, C. R. (2014). Biomarkers for severity of neonatal hypoxic-ischemic encephalopathy and outcomes in newborns receiving hypothermia therapy. J Pediatr, 164(3), 468-474 e461. doi:10.1016/j.jpeds.2013.10.067

Chavez-Valdez, R., Miller, S., Spahic, H., Vaidya, D., Parkinson, C., Dietrick, B., . . . Everett, A. D. (2021). Therapeutic Hypothermia Modulates the Relationships Between Indicators of Severity of Neonatal Hypoxic Ischemic Encephalopathy and Serum Biomarkers. Front Neurol, 12, 748150. doi:10.3389/fneur.2021.748150

Chen, M., Song, H., Cui, J., Johnson, C. E., Hubler, G. K., DePalma, R. G., . . . Xia, W. (2018). Proteomic Profiling of Mouse Brains Exposed to Blast-Induced Mild Traumatic Brain Injury Reveals Changes in Axonal Proteins and Phosphorylated Tau. J Alzheimers Dis, 66(2), 751-773. doi:10.3233/JAD-180726

Chen, Y., Yi, Q., Liu, G., Shen, X., Xuan, L., & Tian, Y. (2013). Cerebral white matter injury and damage to myelin sheath following whole-brain ischemia. Brain Res, 1495, 11-17. doi:10.1016/j.brainres.2012.12.006

Cheong, J. L. Y., Olsen, J. E., Lee, K. J., Spittle, A. J., Opie, G. F., Clark, M., . . . Victorian Infant Collaborative Study, G. (2021). Temporal Trends in Neurodevelopmental Outcomes to 2 Years After Extremely Preterm Birth. JAMA Pediatr, 175(10), 1035-1042. doi:10.1001/jamapediatrics.2021.2052

Chiang, M. C., Lien, R., Chu, S. M., Yang, P. H., Lin, J. J., Hsu, J. F., . . . Lin, K. L. (2016). Serum Lactate, Brain Magnetic Resonance Imaging and Outcome of Neonatal Hypoxic Ischemic Encephalopathy after Therapeutic Hypothermia. Pediatr Neonatol, 57(1), 35-40. doi:10.1016/j.pedneo.2015.04.008

Cho, K. H., Davidson, J. O., Dean, J. M., Bennet, L., & Gunn, A. J. (2020). Cooling and immunomodulation for treating hypoxic-ischemic brain injury. Pediatr Int, 62(7), 770-778. doi:10.1111/ped.14215

Cho, K. H. T., Wassink, G., Galinsky, R., Xu, B., Mathai, S., Dhillon, S. K., . . . Fraser, M. (2019). Protective effects of delayed intraventricular TLR7 agonist administration on cerebral white and gray matter following asphyxia in the preterm fetal sheep. Sci Rep, 9(1), 9562. doi:10.1038/s41598-019-45872-y

Chuang, J. C., & Jones, P. A. (2007). Epigenetics and microRNAs. Pediatr Res, 61(5 Pt 2), 24R-29R. doi:10.1203/pdr.0b013e3180457684

Ciechanowska, A., Ciapala, K., Pawlik, K., Oggioni, M., Mercurio, D., De Simoni, M. G., & Mika, J. (2020). Initiators of Classical and Lectin Complement Pathways Are Differently Engaged after Traumatic Brain Injury-Time-Dependent Changes in the Cortex, Striatum, Thalamus and Hippocampus in a Mouse Model. Int J Mol Sci, 22(1). doi:10.3390/ijms22010045

Cnattingius, S., Johansson, S., & Razaz, N. (2020). Apgar Score and Risk of Neonatal Death among Preterm Infants. N Engl J Med, 383(1), 49-57. doi:10.1056/NEJMoa1915075

Collaborators. (2021, November 18). Human protein atlas. Retrieved from https://www.proteinatlas.org/

Conway, J. M., Walsh, B. H., Boylan, G. B., & Murray, D. M. (2018). Mild hypoxic ischaemic encephalopathy and long term neurodevelopmental outcome - A systematic review. Early Hum Dev, 120, 80-87. doi:10.1016/j.earlhumdev.2018.02.007

Coulthard, L. G., Hawksworth, O. A., Li, R., Balachandran, A., Lee, J. D., Sepehrband, F., . . . Woodruff, T. M. (2017). Complement C5aR1 Signaling Promotes Polarization and Proliferation of Embryonic Neural Progenitor Cells through PKCzeta. J Neurosci, 37(22), 5395-5407. doi:10.1523/JNEUROSCI.0525-17.2017

Counsell, S. J., & Boardman, J. P. (2005). Differential brain growth in the infant born preterm: current knowledge and future developments from brain imaging. Semin Fetal Neonatal Med, 10(5), 403-410. doi:10.1016/j.siny.2005.05.003

Daimon, C. M., Jasien, J. M., Wood, W. H., 3rd, Zhang, Y., Becker, K. G., Silverman, J. L., . . . Maudsley, S. (2015). Hippocampal Transcriptomic and Proteomic Alterations in the BTBR Mouse Model of Autism Spectrum Disorder. Front Physiol, 6, 324. doi:10.3389/fphys.2015.00324

Dammann, O., & Leviton, A. (2007). Perinatal brain damage causation. Dev Neurosci, 29(4-5), 280-288. doi:10.1159/000105469

Dammann, O., & O'Shea, T. M. (2008). Cytokines and perinatal brain damage. Clin Perinatol, 35(4), 643-663, v. doi:10.1016/j.clp.2008.07.011

Danielsson, H., Tebani, A., Zhong, W., Fagerberg, L., Brusselaers, N., Hard, A. L., . . . Hellstrom, A. (2021). Blood protein profiles related to preterm birth and retinopathy of prematurity. Pediatr Res. doi:10.1038/s41390-021-01528-0

de Haan, M., Wyatt, J. S., Roth, S., Vargha-Khadem, F., Gadian, D., & Mishkin, M. (2006). Brain and cognitive-behavioural development after asphyxia at term birth. Dev Sci, 9(4), 350-358. doi:10.1111/j.1467-7687.2006.00499.x

Denihan, N. M., Boylan, G. B., & Murray, D. M. (2015). Metabolomic profiling in perinatal asphyxia: a promising new field. Biomed Res Int, 2015, 254076. doi:10.1155/2015/254076

Denihan, N. M., Kirwan, J. A., Walsh, B. H., Dunn, W. B., Broadhurst, D. I., Boylan, G. B., & Murray, D. M. (2019). Untargeted metabolomic analysis and pathway discovery in perinatal asphyxia and hypoxic-ischaemic encephalopathy. J Cereb Blood Flow Metab, 39(1), 147-162. doi:10.1177/0271678X17726502

Descloux, C., Ginet, V., Clarke, P. G., Puyal, J., & Truttmann, A. C. (2015). Neuronal death after perinatal cerebral hypoxia-ischemia: Focus on autophagy-mediated cell death. Int J Dev Neurosci, 45, 75-85. doi:10.1016/j.ijdevneu.2015.06.008

Dhillon, S. K., Gunn, A. J., Jung, Y., Mathai, S., Bennet, L., & Fraser, M. (2015). Lipopolysaccharide-Induced Preconditioning Attenuates Apoptosis and Differentially Regulates TLR4 and TLR7 Gene Expression after Ischemia in the Preterm Ovine Fetal Brain. Dev Neurosci, 37(6), 497-514. doi:10.1159/000433422

Disdier, C., & Stonestreet, B. S. (2020). Hypoxic-ischemic-related cerebrovascular changes and potential therapeutic strategies in the neonatal brain. J Neurosci Res, 98(7), 1468-1484. doi:10.1002/jnr.24590

Donega, V., van Velthoven, C. T., Nijboer, C. H., van Bel, F., Kas, M. J., Kavelaars, A., & Heijnen, C. J. (2013). Intranasal mesenchymal stem cell treatment for neonatal brain damage: long-term cognitive and sensorimotor improvement. PLoS One, 8(1), e51253. doi:10.1371/journal.pone.0051253

Doyle, L. W., Crowther, C. A., Middleton, P., Marret, S., & Rouse, D. (2009). Magnesium sulphate for women at risk of preterm birth for neuroprotection of the fetus. Cochrane Database Syst Rev(1), CD004661. doi:10.1002/14651858.CD004661.pub3

DuPont, T. L., Chalak, L. F., Morriss, M. C., Burchfield, P. J., Christie, L., & Sanchez, P. J. (2013). Short-term outcomes of newborns with perinatal acidemia who are not eligible for systemic hypothermia therapy. J Pediatr, 162(1), 35-41. doi:10.1016/j.jpeds.2012.06.042

Dzietko, M., Derugin, N., Wendland, M. F., Vexler, Z. S., & Ferriero, D. M. (2013). Delayed VEGF treatment enhances angiogenesis and recovery after neonatal focal rodent stroke. Transl Stroke Res, 4(2), 189-200. doi:10.1007/s12975-012-0221-6

Edwards, A. D., Brocklehurst, P., Gunn, A. J., Halliday, H., Juszczak, E., Levene, M., . . . Azzopardi, D. (2010). Neurological outcomes at 18 months of age after moderate hypothermia for perinatal hypoxic ischaemic encephalopathy: synthesis and meta-analysis of trial data. BMJ, 340, c363. doi:10.1136/bmj.c363

Edwards, A. D., & Tan, S. (2006). Perinatal infections, prematurity and brain injury. Curr Opin Pediatr, 18(2), 119-124. doi:10.1097/01.mop.0000193290.02270.30

Edwards, A. D., Yue, X., Cox, P., Hope, P. L., Azzopardi, D. V., Squier, M. V., & Mehmet, H. (1997). Apoptosis in the brains of infants suffering intrauterine cerebral injury. Pediatr Res, 42(5), 684-689. doi:10.1203/00006450-199711000-00022

Ek, M., Arias, C., Sawchenko, P., & Ericsson-Dahlstrand, A. (2000). Distribution of the EP3 prostaglandin E(2) receptor subtype in the rat brain: relationship to sites of interleukin-1-induced cellular responsiveness. J Comp Neurol, 428(1), 5-20. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/11058221

Ek, M., Engblom, D., Saha, S., Blomqvist, A., Jakobsson, P. J., & Ericsson-Dahlstrand, A. (2001). Inflammatory response: pathway across the blood-brain barrier. Nature, 410(6827), 430-431. doi:10.1038/35068632

El Farargy, M. S., & Soliman, N. A. (2019). A randomized controlled trial on the use of magnesium sulfate and melatonin in neonatal hypoxic ischemic encephalopathy. J Neonatal Perinatal Med, 12(4), 379-384. doi:10.3233/NPM-181830

El-Dib, M., Inder, T. E., Chalak, L. F., Massaro, A. N., Thoresen, M., & Gunn, A. J. (2019). Should therapeutic hypothermia be offered to babies with mild neonatal encephalopathy in the first 6 h after birth? Pediatr Res, 85(4), 442-448. doi:10.1038/s41390-019-0291-1

Espana, L. Y., Lee, R. M., Ling, J. M., Jeromin, A., Mayer, A. R., & Meier, T. B. (2017). Serial Assessment of Gray Matter Abnormalities after Sport-Related Concussion. J Neurotrauma, 34(22), 3143-3152. doi:10.1089/neu.2017.5002

Etemadikhah, M., Niazi, A., Wetterberg, L., & Feuk, L. (2020). Transcriptome analysis of fibroblasts from schizophrenia patients reveals differential expression of schizophrenia-related genes. Sci Rep, 10(1), 630. doi:10.1038/s41598-020-57467-z

Evans, A. C., Karama, S., Vasung, L., & Iturria-Medina, Y. (2017). Understanding brain development: a major step. Lancet Neurol, 16(3), 178-179. doi:10.1016/S1474-4422(16)30232-0

Falck, M., Osredkar, D., Maes, E., Flatebo, T., Wood, T. R., Sabir, H., & Thoresen, M. (2017). Hypothermic Neuronal Rescue from Infection-Sensitised Hypoxic-Ischaemic Brain Injury Is Pathogen Dependent. Dev Neurosci, 39(1-4), 238-247. doi:10.1159/000455838

Falck, M., Osredkar, D., Wood, T. R., Maes, E., Flatebo, T., Sabir, H., & Thoresen, M. (2018). Neonatal Systemic Inflammation Induces Inflammatory Reactions and Brain Apoptosis in a Pathogen-Specific Manner. Neonatology, 113(3), 212-220. doi:10.1159/000481980

Fang, M., Jiang, H., Ye, L., Cai, C., Hu, Y., Pan, S., . . . Lin, Z. (2017). Metformin treatment after the hypoxia-ischemia attenuates brain injury in newborn rats. Oncotarget, 8(43), 75308-75325. doi:10.18632/oncotarget.20779

Fang, X. X., Jiang, X. L., Han, X. H., Peng, Y. P., & Qiu, Y. H. (2013). Neuroprotection of interleukin-6 against NMDA-induced neurotoxicity is mediated by JAK/STAT3, MAPK/ERK, and PI3K/AKT signaling pathways. Cell Mol Neurobiol, 33(2), 241-251. doi:10.1007/s10571-012-9891-6

Fauchere, J. C., Koller, B. M., Tschopp, A., Dame, C., Ruegger, C., Bucher, H. U., & Swiss Erythropoietin Neuroprotection Trial, G. (2015). Safety of Early High-Dose Recombinant Erythropoietin for Neuroprotection in Very Preterm Infants. J Pediatr, 167(1), 52-57 e51-53. doi:10.1016/j.jpeds.2015.02.052

Ferber, A., Minior, V. K., Bornstein, E., & Divon, M. Y. (2005). Fetal "nonreassuring status" is associated with elevation of nucleated red blood cell counts and interleukin-6. Am J Obstet Gynecol, 192(5), 1427-1429. doi:10.1016/j.ajog.2004.12.076

Fernandez-Lopez, D., Pazos, M. R., Tolon, R. M., Moro, M. A., Romero, J., Lizasoain, I., & Martinez-Orgado, J. (2007). The cannabinoid agonist WIN55212 reduces brain damage in an in vivo model of hypoxic-ischemic encephalopathy in newborn rats. Pediatr Res, 62(3), 255-260. doi:10.1203/PDR.0b013e318123fbb8

Ferrari, D. C., Nesic, O., & Perez-Polo, J. R. (2010). Perspectives on neonatal hypoxia/ischemia-induced edema formation. Neurochem Res, 35(12), 1957-1965. doi:10.1007/s11064-010-0308-y

Ferriero, D. M. (2001). Oxidant mechanisms in neonatal hypoxia-ischemia. Dev Neurosci, 23(3), 198-202. doi:10.1159/000046143

Fiala, M., Baumert, M., Surmiak, P., Walencka, Z., & Sodowska, P. (2016). Umbilical markers of perinatal hypoxia. Ginekol Pol, 87(3), 200-204. doi:10.17772/gp/60552

Filippi, L., Fiorini, P., Catarzi, S., Berti, E., Padrini, L., Landucci, E., . . . Guerrini, R. (2018). Safety and efficacy of topiramate in neonates with hypoxic ischemic encephalopathy treated with hypothermia (NeoNATI): a feasibility study. J Matern Fetal Neonatal Med, 31(8), 973-980. doi:10.1080/14767058.2017.1304536

Finder, M., Boylan, G. B., Twomey, D., Ahearne, C., Murray, D. M., & Hallberg, B. (2020). Two-Year Neurodevelopmental Outcomes After Mild Hypoxic Ischemic Encephalopathy in the Era of Therapeutic Hypothermia. JAMA Pediatr, 174(1), 48-55. doi:10.1001/jamapediatrics.2019.4011

Finer, N. N., Higgins, R., Kattwinkel, J., & Martin, R. J. (2006). Summary proceedings from the apnea-of-prematurity group. Pediatrics, 117(3 Pt 2), S47-51. doi:10.1542/peds.2005-0620H

Fleiss, B., & Gressens, P. (2012). Tertiary mechanisms of brain damage: a new hope for treatment of cerebral palsy? Lancet Neurol, 11(6), 556-566. doi:10.1016/S1474-4422(12)70058-3

Fleiss, B., Rivkees, S. A., & Gressens, P. (2019). Early origins of neuropsychiatric disorders. Pediatr Res, 85(2), 113-114. doi:10.1038/s41390-018-0225-3

Fleiss, B., Tann, C. J., Degos, V., Sigaut, S., Van Steenwinckel, J., Schang, A. L., . . . Gressens, P. (2015). Inflammation-induced sensitization of the brain in term infants. Dev Med Child Neurol, 57 Suppl 3, 17-28. doi:10.1111/dmcn.12723

Florio, P., Abella, R., Marinoni, E., Di Iorio, R., Li Volti, G., Galvano, F., . . . Gazzolo, D. (2010). Biochemical markers of perinatal brain damage. Front Biosci (Schol Ed), 2, 47-72. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/20036928

Florio, P., Luisi, S., Bruschettini, M., Grutzfeld, D., Dobrzanska, A., Bruschettini, P., . . . Gazzolo, D. (2004). Cerebrospinal fluid activin a measurement in asphyxiated full-term newborns predicts hypoxic ischemic encephalopathy. Clin Chem, 50(12), 2386-2389. doi:10.1373/clinchem.2004.035774

Follett, P. L., Deng, W., Dai, W., Talos, D. M., Massillon, L. J., Rosenberg, P. A., . . . Jensen, F. E. (2004). Glutamate receptor-mediated oligodendrocyte toxicity in periventricular leukomalacia: a protective role for topiramate. J Neurosci, 24(18), 4412-4420. doi:10.1523/JNEUROSCI.0477-04.2004

Friauf, E., & Shatz, C. J. (1991). Changing patterns of synaptic input to subplate and cortical plate during development of visual cortex. J Neurophysiol, 66(6), 2059-2071. doi:10.1152/jn.1991.66.6.2059

Fromer, M., Pocklington, A. J., Kavanagh, D. H., Williams, H. J., Dwyer, S., Gormley, P., . . . O'Donovan, M. C. (2014). De novo mutations in schizophrenia implicate synaptic networks. Nature, 506(7487), 179-184. doi:10.1038/nature12929

Galinsky, R., Dean, J. M., Lingam, I., Robertson, N. J., Mallard, C., Bennet, L., & Gunn, A. J. (2020). A Systematic Review of Magnesium Sulfate for Perinatal Neuroprotection: What Have We Learnt From the Past Decade? Front Neurol, 11, 449. doi:10.3389/fneur.2020.00449

Galluzzi, L., Blomgren, K., & Kroemer, G. (2009). Mitochondrial membrane permeabilization in neuronal injury. Nat Rev Neurosci, 10(7), 481-494. doi:10.1038/nrn2665

Gano, D., Ho, M. L., Partridge, J. C., Glass, H. C., Xu, D., Barkovich, A. J., & Ferriero, D. M. (2016). Antenatal Exposure to Magnesium Sulfate Is Associated with Reduced Cerebellar Hemorrhage in Preterm Newborns. J Pediatr, 178, 68-74. doi:10.1016/j.jpeds.2016.06.053

Garcia-Alix, A., Cabanas, F., Pellicer, A., Hernanz, A., Stiris, T. A., & Quero, J. (1994). Neuron-specific enolase and myelin basic protein: relationship of cerebrospinal fluid concentrations to the neurologic condition of asphyxiated full-term infants. Pediatrics, 93(2), 234-240. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/7510064

Garcia-Marin, V., Blazquez-Llorca, L., Rodriguez, J. R., Gonzalez-Soriano, J., & DeFelipe, J. (2010). Differential distribution of neurons in the gyral white matter of the human cerebral cortex. J Comp Neurol, 518(23), 4740-4759. doi:10.1002/cne.22485

Gilles, F., Gressens, P., Dammann, O., & Leviton, A. (2018). Hypoxia-ischemia is not an antecedent of most preterm brain damage: the illusion of validity. Dev Med Child Neurol, 60(2), 120-125. doi:10.1111/dmcn.13483

Giza, C. C., & Prins, M. L. (2006). Is being plastic fantastic? Mechanisms of altered plasticity after developmental traumatic brain injury. Dev Neurosci, 28(4-5), 364-379. doi:10.1159/000094163

Glass, H. C., Costarino, A. T., Stayer, S. A., Brett, C. M., Cladis, F., & Davis, P. J. (2015). Outcomes for extremely premature infants. Anesth Analg, 120(6), 1337-1351. doi:10.1213/ANE.0000000000000705

Gong, L., Tang, Y., An, R., Lin, M., Chen, L., & Du, J. (2017). RTN1-C mediates cerebral ischemia/reperfusion injury via ER stress and mitochondria-associated apoptosis pathways. Cell Death Dis, 8(10), e3080. doi:10.1038/cddis.2017.465

Gozdas, E., Parikh, N. A., Merhar, S. L., Tkach, J. A., He, L., & Holland, S. K. (2018). Altered functional network connectivity in preterm infants: antecedents of cognitive and motor impairments? Brain Struct Funct, 223(8), 3665-3680. doi:10.1007/s00429-018-1707-0

Graham, E. M., Everett, A. D., Delpech, J. C., & Northington, F. J. (2018). Blood biomarkers for evaluation of perinatal encephalopathy: state of the art. Curr Opin Pediatr, 30(2), 199-203. doi:10.1097/MOP.0000000000000591

Graham, E. M., Ruis, K. A., Hartman, A. L., Northington, F. J., & Fox, H. E. (2008). A systematic review of the role of intrapartum hypoxia-ischemia in the causation of neonatal encephalopathy. Am J Obstet Gynecol, 199(6), 587-595. doi:10.1016/j.ajog.2008.06.094

Graham, E. M., Sheldon, R. A., Flock, D. L., Ferriero, D. M., Martin, L. J., O'Riordan, D. P., & Northington, F. J. (2004). Neonatal mice lacking functional Fas death receptors are resistant to hypoxic-ischemic brain injury. Neurobiol Dis, 17(1), 89-98. doi:10.1016/j.nbd.2004.05.007

Gunn, A. J., Gunn, T. R., de Haan, H. H., Williams, C. E., & Gluckman, P. D. (1997). Dramatic neuronal rescue with prolonged selective head cooling after ischemia in fetal lambs. J Clin Invest, 99(2), 248-256. doi:10.1172/JCI119153

Guo, H., Zhou, H., Lu, J., Qu, Y., Yu, D., & Tong, Y. (2016). Vascular endothelial growth factor: an attractive target in the treatment of hypoxic/ischemic brain injury. Neural Regen Res, 11(1), 174-179. doi:10.4103/1673-5374.175067

H.C., K., & D.L., A. (2002). Neuropathology. In (7th ed., Vol. 1, pp. 519-606). London: Arnold Publishers

Hagberg, H., David Edwards, A., & Groenendaal, F. (2016). Perinatal brain damage: The term infant. Neurobiol Dis, 92(Pt A), 102-112. doi:10.1016/j.nbd.2015.09.011

Hagberg, H., Gilland, E., Diemer, N. H., & Andine, P. (1994). Hypoxia-ischemia in the neonatal rat brain: histopathology after post-treatment with NMDA and non-NMDA receptor antagonists. Biol Neonate, 66(4), 205-213. doi:10.1159/000244109

Hagberg, H., Mallard, C., Ferriero, D. M., Vannucci, S. J., Levison, S. W., Vexler, Z. S., & Gressens, P. (2015). The role of inflammation in perinatal brain injury. Nat Rev Neurol, 11(4), 192-208. doi:10.1038/nrneurol.2015.13

Hagberg, H., Mallard, C., Rousset, C. I., & Thornton, C. (2014). Mitochondria: hub of injury responses in the developing brain. Lancet Neurol, 13(2), 217-232. doi:10.1016/S1474-4422(13)70261-8

Hakim, S. M., Ammar, M. A., & Reyad, M. S. (2018). Effect of therapeutic hypothermia on survival and neurological outcome in adults suffering cardiac arrest: a systematic review and meta-analysis. Minerva Anestesiol, 84(6), 720-730. doi:10.23736/S0375-9393.18.12164-X

Halford, J., Shen, S., Itamura, K., Levine, J., Chong, A. C., Czerwieniec, G., . . . Wanner, I. B. (2017). New astroglial injury-defined biomarkers for neurotrauma assessment. J Cereb Blood Flow Metab, 37(10), 3278-3299. doi:10.1177/0271678X17724681

Halliwell, B. (1992). Reactive oxygen species and the central nervous system. J Neurochem, 59(5), 1609-1623. doi:10.1111/j.1471-4159.1992.tb10990.x

Hammond, T. R., Robinton, D., & Stevens, B. (2018). Microglia and the Brain: Complementary Partners in Development and Disease. Annu Rev Cell Dev Biol, 34, 523-544. doi:10.1146/annurev-cellbio-100616-060509

Hankins, G. D., & Speer, M. (2003). Defining the pathogenesis and pathophysiology of neonatal encephalopathy and cerebral palsy. Obstet Gynecol, 102(3), 628-636. doi:10.1016/s0029-7844(03)00574-x

Harding, D. (2007). Impact of common genetic variation on neonatal disease and outcome. Arch Dis Child Fetal Neonatal Ed, 92(5), F408-413. doi:10.1136/adc.2006.108670

Harper, M. M., Rudd, D., Meyer, K. J., Kanthasamy, A. G., Anantharam, V., Pieper, A. A., . . . John, M. (2020). Identification of chronic brain protein changes and protein targets of serum auto-antibodies after blast-mediated traumatic brain injury. Heliyon, 6(2), e03374. doi:10.1016/j.heliyon.2020.e03374

Harris, S. E., Cox, S. R., Bell, S., Marioni, R. E., Prins, B. P., Pattie, A., . . . Deary, I. J. (2020). Neurology-related protein biomarkers are associated with cognitive ability and brain volume in older age. Nat Commun, 11(1), 800. doi:10.1038/s41467-019-14161-7

Helenius, K., Sjors, G., Shah, P. S., Modi, N., Reichman, B., Morisaki, N., . . . International Network for Evaluating Outcomes of, N. (2017). Survival in Very Preterm Infants: An International Comparison of 10 National Neonatal Networks. Pediatrics, 140(6). doi:10.1542/peds.2017-1264

Hellstrom, A., Engstrom, E., Hard, A. L., Albertsson-Wikland, K., Carlsson, B., Niklasson, A., . . . Smith, L. E. (2003). Postnatal serum insulin-like growth factor I deficiency is associated with retinopathy of prematurity and other complications of premature birth. Pediatrics, 112(5), 1016-1020. doi:10.1542/peds.112.5.1016

Herlenius, E. (2011). An inflammatory pathway to apnea and autonomic dysregulation. Respir Physiol Neurobiol, 178(3), 449-457. doi:10.1016/j.resp.2011.06.026

Herrera, T. I., Edwards, L., Malcolm, W. F., Smith, P. B., Fisher, K. A., Pizoli, C., . . . Bidegain, M. (2018). Outcomes of preterm infants treated with hypothermia for hypoxic-ischemic encephalopathy. Early Hum Dev, 125, 1-7. doi:10.1016/j.earlhumdev.2018.08.003

Herrera-Marschitz, M., Neira-Pena, T., Rojas-Mancilla, E., Espina-Marchant, P., Esmar, D., Perez, R., . . . Gebicke-Haerter, P. J. (2014). Perinatal asphyxia: CNS development and deficits with delayed onset. Front Neurosci, 8, 47. doi:10.3389/fnins.2014.00047

Herz, J., Koster, C., Reinboth, B. S., Dzietko, M., Hansen, W., Sabir, H., . . . Felderhoff-Muser, U. (2018). Interaction between hypothermia and delayed mesenchymal stem cell therapy in neonatal hypoxic-ischemic brain injury. Brain Behav Immun, 70, 118-130. doi:10.1016/j.bbi.2018.02.006

Higgins, R. D., & Shankaran, S. (2011). Hypothermia: novel approaches for premature infants. Early Hum Dev, 87 Suppl 1, S17-18. doi:10.1016/j.earlhumdev.2011.01.004

Hirtz, D. G., Weiner, S. J., Bulas, D., DiPietro, M., Seibert, J., Rouse, D. J., . . . Human Development Maternal-Fetal Medicine Units, N. (2015). Antenatal Magnesium and Cerebral Palsy in Preterm Infants. J Pediatr, 167(4), 834-839 e833. doi:10.1016/j.jpeds.2015.06.067

Hoerder-Suabedissen, A., & Molnar, Z. (2015). Development, evolution and pathology of neocortical subplate neurons. Nat Rev Neurosci, 16(3), 133-146. doi:10.1038/nrn3915

Hofstetter, A. O., Legnevall, L., Herlenius, E., & Katz-Salamon, M. (2008). Cardiorespiratory development in extremely preterm infants: vulnerability to infection and persistence of events beyond term-equivalent age. Acta Paediatr, 97(3), 285-292. doi:10.1111/j.1651-2227.2007.00618.x

Hofstetter, A. O., Saha, S., Siljehav, V., Jakobsson, P. J., & Herlenius, E. (2007). The induced prostaglandin E2 pathway is a key regulator of the respiratory response to infection and hypoxia in neonates. Proc Natl Acad Sci U S A, 104(23), 9894-9899. doi:10.1073/pnas.0611468104

Hofstetter, C., Boost, K. A., Flondor, M., Basagan-Mogol, E., Betz, C., Homann, M., . . . Zwissler, B. (2007). Anti-inflammatory effects of sevoflurane and mild hypothermia in endotoxemic rats. Acta Anaesthesiol Scand, 51(7), 893-899. doi:10.1111/j.1399-6576.2007.01353.x

Hu, X. L., Chen, G., Zhang, S., Zheng, J., Wu, J., Bai, Q. R., . . . Shen, Q. (2017). Persistent Expression of VCAM1 in Radial Glial Cells Is Required for the Embryonic Origin of Postnatal Neural Stem Cells. Neuron, 95(2), 309-325 e306. doi:10.1016/j.neuron.2017.06.047

Huang, R., Zhang, J., Ren, C., Zhang, X., Gu, L., Dong, Y., . . . Zhang, J. (2019). Effect of erythropoietin on Fas/FasL expression in brain tissues of neonatal rats with hypoxic-ischemic brain damage. Neuroreport, 30(4), 262-268. doi:10.1097/WNR.0000000000001194

Hubbard, J. A., Szu, J. I., & Binder, D. K. (2018). The role of aquaporin-4 in synaptic plasticity, memory and disease. Brain Res Bull, 136, 118-129. doi:10.1016/j.brainresbull.2017.02.011

Huet, O., Kinirons, B., Dupic, L., Lajeunie, E., Mazoit, J. X., Benhamou, D., . . . Duranteau, J. (2007). Induced mild hypothermia reduces mortality during acute inflammation in rats. Acta Anaesthesiol Scand, 51(9), 1211-1216. doi:10.1111/j.1399-6576.2007.01419.x

Hutchinson, E. A., De Luca, C. R., Doyle, L. W., Roberts, G., Anderson, P. J., & Victorian Infant Collaborative Study, G. (2013). School-age outcomes of extremely preterm or extremely low birth weight children. Pediatrics, 131(4), e1053-1061. doi:10.1542/peds.2012-2311

Inder, T., Neil, J., Kroenke, C., Dieni, S., Yoder, B., & Rees, S. (2005). Investigation of cerebral development and injury in the prematurely born primate by magnetic resonance imaging and histopathology. Dev Neurosci, 27(2-4), 100-111. doi:10.1159/000085981

Inder, T. E., & Volpe, J. J. (2000). Mechanisms of perinatal brain injury. Semin Neonatol, 5(1), 3-16. doi:10.1053/siny.1999.0112

Inta, D., Alfonso, J., von Engelhardt, J., Kreuzberg, M. M., Meyer, A. H., van Hooft, J. A., & Monyer, H. (2008). Neurogenesis and widespread forebrain migration of distinct GABAergic neurons from the postnatal subventricular zone. Proc Natl Acad Sci U S A, 105(52), 20994-20999. doi:10.1073/pnas.0807059105

Ismail, F. Y., Fatemi, A., & Johnston, M. V. (2017). Cerebral plasticity: Windows of opportunity in the developing brain. Eur J Paediatr Neurol, 21(1), 23-48. doi:10.1016/j.ejpn.2016.07.007

Ivain, P., Montaldo, P., Khan, A., Elagovan, R., Burgod, C., Morales, M. M., . . . Thayyil, S. (2021). Erythropoietin monotherapy for neuroprotection after neonatal encephalopathy in low-to-middle income countries: a systematic review and meta-analysis. J Perinatol, 41(9), 2134-2140. doi:10.1038/s41372-021-01132-4

Jacobs, S. E., Berg, M., Hunt, R., Tarnow-Mordi, W. O., Inder, T. E., & Davis, P. G. (2013). Cooling for newborns with hypoxic ischaemic encephalopathy. Cochrane Database Syst Rev(1), CD003311. doi:10.1002/14651858.CD003311.pub3

Jain, P., Spaeder, M. C., Donofrio, M. T., Sinha, P., Jonas, R. A., & Levy, R. J. (2014). Detection of alpha II-spectrin breakdown products in the serum of neonates with congenital heart disease*. Pediatr Crit Care Med, 15(3), 229-235. doi:10.1097/PCC.0000000000000059

Janvier, A., Khairy, M., Kokkotis, A., Cormier, C., Messmer, D., & Barrington, K. J. (2004). Apnea is associated with neurodevelopmental impairment in very low birth weight infants. J Perinatol, 24(12), 763-768. doi:10.1038/sj.jp.7211182

Jenkins, D. D., Rollins, L. G., Perkel, J. K., Wagner, C. L., Katikaneni, L. P., Bass, W. T., . . . Martin, R. H. (2012). Serum cytokines in a clinical trial of hypothermia for neonatal hypoxic-ischemic encephalopathy. J Cereb Blood Flow Metab, 32(10), 1888-1896. doi:10.1038/jcbfm.2012.83

Jessen, N. A., Munk, A. S., Lundgaard, I., & Nedergaard, M. (2015). The Glymphatic System: A Beginner's Guide. Neurochem Res, 40(12), 2583-2599. doi:10.1007/s11064-015-1581-6

Jiao, B., Liu, H., Guo, L., Liao, X., Zhou, Y., Weng, L., . . . Shen, L. (2021). Performance of Plasma Amyloid beta, Total Tau, and Neurofilament Light Chain in the Identification of Probable Alzheimer's Disease in South China. Front Aging Neurosci, 13, 749649. doi:10.3389/fnagi.2021.749649

Johnson, S., & Marlow, N. (2011). Preterm birth and childhood psychiatric disorders. Pediatr Res, 69(5 Pt 2), 11R-18R. doi:10.1203/PDR.0b013e318212faa0

Johnson, S., & Marlow, N. (2017). Early and long-term outcome of infants born extremely preterm. Arch Dis Child, 102(1), 97-102. doi:10.1136/archdischild-2015-309581

Johnston, M. V. (2005). Excitotoxicity in perinatal brain injury. Brain Pathol, 15(3), 234-240. doi:10.1111/j.1750-3639.2005.tb00526.x

Johnston, M. V. (2009). Plasticity in the developing brain: implications for rehabilitation. Dev Disabil Res Rev, 15(2), 94-101. doi:10.1002/ddrr.64

Johnston, M. V., Ishida, A., Ishida, W. N., Matsushita, H. B., Nishimura, A., & Tsuji, M. (2009). Plasticity and injury in the developing brain. Brain Dev, 31(1), 1-10. doi:10.1016/j.braindev.2008.03.014

Johnston, M. V., Nakajima, W., & Hagberg, H. (2002). Mechanisms of hypoxic neurodegeneration in the developing brain. Neuroscientist, 8(3), 212-220. doi:10.1177/1073858402008003007

Ju, S., Xu, C., Wang, G., & Zhang, L. (2019). VEGF-C Induces Alternative Activation of Microglia to Promote Recovery from Traumatic Brain Injury. J Alzheimers Dis, 68(4), 1687-1697. doi:10.3233/JAD-190063

Juul, S. E., Comstock, B. A., Heagerty, P. J., Mayock, D. E., Goodman, A. M., Hauge, S., . . . Wu, Y. W. (2018). High-Dose Erythropoietin for Asphyxia and Encephalopathy (HEAL): A Randomized Controlled Trial - Background, Aims, and Study Protocol. Neonatology, 113(4), 331-338. doi:10.1159/000486820

Juul, S. E., Comstock, B. A., Wadhawan, R., Mayock, D. E., Courtney, S. E., Robinson, T., . . . Consortium, P. T. (2020). A Randomized Trial of Erythropoietin for Neuroprotection in Preterm Infants. N Engl J Med, 382(3), 233-243. doi:10.1056/NEJMoa1907423

Juul, S. E., & Ferriero, D. M. (2014). Pharmacologic neuroprotective strategies in neonatal brain injury. Clin Perinatol, 41(1), 119-131. doi:10.1016/j.clp.2013.09.004

Kaandorp, J. J., van Bel, F., Veen, S., Derks, J. B., Groenendaal, F., Rijken, M., . . . Benders, M. J. (2012). Long-term neuroprotective effects of allopurinol after moderate perinatal asphyxia: follow-up of two randomised controlled trials. Arch Dis Child Fetal Neonatal Ed, 97(3), F162-166. doi:10.1136/archdischild-2011-300356

Karnatovskaia, L. V., Wartenberg, K. E., & Freeman, W. D. (2014). Therapeutic hypothermia for neuroprotection: history, mechanisms, risks, and clinical applications. Neurohospitalist, 4(3), 153-163. doi:10.1177/1941874413519802

Katano, T., Takao, K., Abe, M., Yamazaki, M., Watanabe, M., Miyakawa, T., . . . Ito, S. (2018). Distribution of Caskin1 protein and phenotypic characterization of its knockout mice using a comprehensive behavioral test battery. Mol Brain, 11(1), 63. doi:10.1186/s13041-018-0407-2

Kawano, T., Anrather, J., Zhou, P., Park, L., Wang, G., Frys, K. A., . . . Iadecola, C. (2006). Prostaglandin E2 EP1 receptors: downstream effectors of COX-2 neurotoxicity. Nat Med, 12(2), 225-229. doi:10.1038/nm1362

Kelen, D., Andorka, C., Szabo, M., Alafuzoff, A., Kaila, K., & Summanen, M. (2017). Serum copeptin and neuron specific enolase are markers of neonatal distress and long-term neurodevelopmental outcome. PLoS One, 12(9), e0184593. doi:10.1371/journal.pone.0184593

Kelly, R., Ramaiah, S. M., Sheridan, H., Cruickshank, H., Rudnicka, M., Kissack, C., . . . Stenson, B. J. (2018). Dose-dependent relationship between acidosis at birth and likelihood of death or cerebral palsy. Arch Dis Child Fetal Neonatal Ed, 103(6), F567-F572. doi:10.1136/archdischild-2017-314034

Kerr-Wilson, C. O., Mackay, D. F., Smith, G. C., & Pell, J. P. (2012). Meta-analysis of the association between preterm delivery and intelligence. J Public Health (Oxf), 34(2), 209-216. doi:10.1093/pubmed/fdr024

Kerstjens, J. M., de Winter, A. F., Bocca-Tjeertes, I. F., Bos, A. F., & Reijneveld, S. A. (2012). Risk of developmental delay increases exponentially as gestational age of preterm infants decreases: a cohort study at age 4 years. Dev Med Child Neurol, 54(12), 1096-1101. doi:10.1111/j.1469-8749.2012.04423.x

King, M. R., Anderson, N. J., Deciu, M., Guernsey, L. S., Cundiff, M., Hajizadeh, S., & Jolivalt, C. G. (2020). Insulin deficiency, but not resistance, exaggerates cognitive deficits in transgenic mice expressing human amyloid and tau proteins. Reversal by Exendin-4 treatment. J Neurosci Res, 98(11), 2357-2369. doi:10.1002/jnr.24706

Kinney, H. C. (2009). The encephalopathy of prematurity: one pediatric neuropathologist's perspective. Semin Pediatr Neurol, 16(4), 179-190. doi:10.1016/j.spen.2009.09.003

Koehler, R. C., Yang, Z. J., Lee, J. K., & Martin, L. J. (2018). Perinatal hypoxic-ischemic brain injury in large animal models: Relevance to human neonatal encephalopathy. J Cereb Blood Flow Metab, 38(12), 2092-2111. doi:10.1177/0271678X18797328

Kondo, S., Al-Hasani, H., Hoerder-Suabedissen, A., Wang, W. Z., & Molnar, Z. (2015). Secretory function in subplate neurons during cortical development. Front Neurosci, 9, 100. doi:10.3389/fnins.2015.00100

Koning, G., Leverin, A. L., Nair, S., Schwendimann, L., Ek, J., Carlsson, Y., . . . Hagberg, H. (2019). Magnesium induces preconditioning of the neonatal brain via profound mitochondrial protection. J Cereb Blood Flow Metab, 39(6), 1038-1055. doi:10.1177/0271678X17746132

Koning, G., Lyngfelt, E., Svedin, P., Leverin, A. L., Jinnai, M., Gressens, P., . . . Hagberg, H. (2018). Magnesium sulphate induces preconditioning in preterm rodent models of cerebral hypoxia-ischemia. Int J Dev Neurosci, 70, 56-66. doi:10.1016/j.ijdevneu.2018.01.002

Kostovic, I., & Jovanov-Milosevic, N. (2006). The development of cerebral connections during the first 20-45 weeks' gestation. Semin Fetal Neonatal Med, 11(6), 415-422. doi:10.1016/j.siny.2006.07.001

Kostovic, I., & Judas, M. (2006). Prolonged coexistence of transient and permanent circuitry elements in the developing cerebral cortex of fetuses and preterm infants. Dev Med Child Neurol, 48(5), 388-393. doi:10.1017/S0012162206000831

Kostovic, I., Judas, M., & Sedmak, G. (2011). Developmental history of the subplate zone, subplate neurons and interstitial white matter neurons: relevance for schizophrenia. Int J Dev Neurosci, 29(3), 193-205. doi:10.1016/j.ijdevneu.2010.09.005

Kress, B. T., Iliff, J. J., Xia, M., Wang, M., Wei, H. S., Zeppenfeld, D., . . . Nedergaard, M. (2014). Impairment of paravascular clearance pathways in the aging brain. Ann Neurol, 76(6), 845-861. doi:10.1002/ana.24271

Kuban, K. C. K., Heeren, T., O'Shea, T. M., Joseph, R. M., Fichorova, R. N., Douglass, L., . . . Investigators, E. S. (2018). Among Children Born Extremely Preterm a Higher Level of Circulating Neurotrophins Is Associated with Lower Risk of Cognitive Impairment at School Age. J Pediatr, 201, 40-48 e44. doi:10.1016/j.jpeds.2018.05.021

Kuban, K. C. K., Jara, H., O'Shea, T. M., Heeren, T., Joseph, R. M., Fichorova, R. N., . . . Investigators, E. S. (2019). Association of Circulating Proinflammatory and Anti-inflammatory Protein Biomarkers in Extremely Preterm Born Children with Subsequent Brain Magnetic Resonance Imaging Volumes and Cognitive Function at Age 10 Years. J Pediatr, 210, 81-90 e83. doi:10.1016/j.jpeds.2019.03.018

Kurinczuk, J. J., White-Koning, M., & Badawi, N. (2010). Epidemiology of neonatal encephalopathy and hypoxic-ischaemic encephalopathy. Early Hum Dev, 86(6), 329-338. doi:10.1016/j.earlhumdev.2010.05.010

Lafuente, H., Pazos, M. R., Alvarez, A., Mohammed, N., Santos, M., Arizti, M., . . . Martinez-Orgado, J. A. (2016). Effects of Cannabidiol and Hypothermia on Short-Term Brain Damage in New-Born Piglets after Acute Hypoxia-Ischemia. Front Neurosci, 10, 323. doi:10.3389/fnins.2016.00323

Larpthaveesarp, A., Ferriero, D. M., & Gonzalez, F. F. (2015). Growth factors for the treatment of ischemic brain injury (growth factor treatment). Brain Sci, 5(2), 165-177. doi:10.3390/brainsci5020165

Le Bras, B., Barallobre, M. J., Homman-Ludiye, J., Ny, A., Wyns, S., Tammela, T., . . . Thomas, J. L. (2006). VEGF-C is a trophic factor for neural progenitors in the vertebrate embryonic brain. Nat Neurosci, 9(3), 340-348. doi:10.1038/nn1646

Lechpammer, M., Manning, S. M., Samonte, F., Nelligan, J., Sabo, E., Talos, D. M., . . . Jensen, F. E. (2008). Minocycline treatment following hypoxic/ischaemic injury attenuates white matter injury in a rodent model of periventricular leucomalacia. Neuropathol Appl Neurobiol, 34(4), 379-393. doi:10.1111/j.1365-2990.2007.00925.x

Lee, W. L. A., Michael-Titus, A. T., & Shah, D. K. (2017). Hypoxic-Ischaemic Encephalopathy and the Blood-Brain Barrier in Neonates. Dev Neurosci, 39(1-4), 49-58. doi:10.1159/000467392

Lefevre, J., Germanaud, D., Dubois, J., Rousseau, F., de Macedo Santos, I., Angleys, H., . . . De Guio, F. (2016). Are Developmental Trajectories of Cortical Folding Comparable Between Cross-sectional Datasets of Fetuses and Preterm Newborns? Cerebral Cortex, 26(7), 3023-3035. doi:10.1093/cercor/bhv123

Leifsdottir, K., Mehmet, H., Eksborg, S., & Herlenius, E. (2018). Fas-ligand and interleukin-6 in the cerebrospinal fluid are early predictors of hypoxic-ischemic encephalopathy and long-term outcomes after birth asphyxia in term infants. J Neuroinflammation, 15(1), 223. doi:10.1186/s12974-018-1253-y

Leng, L. (2017). Hypothermia Therapy after Traumatic Brain Injury: A Systematic Review and Meta-Analysis. Turk Neurosurg. doi:10.5137/1019-5149.JTN.19696-16.2

Levene, M. I., Sands, C., Grindulis, H., & Moore, J. R. (1986). Comparison of two methods of predicting outcome in perinatal asphyxia. Lancet, 1(8472), 67-69. doi:10.1016/s0140-6736(86)90718-x

Ley, D., Hallberg, B., Hansen-Pupp, I., Dani, C., Ramenghi, L. A., Marlow, N., . . . study, t. (2019). rhIGF-1/rhIGFBP-3 in Preterm Infants: A Phase 2 Randomized Controlled Trial. J Pediatr, 206, 56-65 e58. doi:10.1016/j.jpeds.2018.10.033

Li, A. M., Chau, V., Poskitt, K. J., Sargent, M. A., Lupton, B. A., Hill, A., . . . Miller, S. P. (2009). White matter injury in term newborns with neonatal encephalopathy. Pediatr Res, 65(1), 85-89. doi:10.1203/PDR.0b013e31818912d2

Li, B., Concepcion, K., Meng, X., & Zhang, L. (2017). Brain-immune interactions in perinatal hypoxic-ischemic brain injury. Prog Neurobiol, 159, 50-68. doi:10.1016/j.pneurobio.2017.10.006

Li, H., Zhu, H., Xu, C. J., & Yuan, J. (1998). Cleavage of BID by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell, 94(4), 491-501. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/9727492

Li, J., An, Y., Wang, J. N., Yin, X. P., Zhou, H., & Wang, Y. S. (2020). Curcumin targets vascular endothelial growth factor via activating the PI3K/Akt signaling pathway and improves brain hypoxic-ischemic injury in neonatal rats. Korean J Physiol Pharmacol, 24(5), 423-431. doi:10.4196/kjpp.2020.24.5.423

Li, W. X., Li, G. H., Tong, X., Yang, P. P., Huang, J. F., Xu, L., & Dai, S. X. (2020). Systematic metabolic analysis of potential target, therapeutic drug, diagnostic method and animal model applicability in three neurodegenerative diseases. Aging (Albany NY), 12(10), 9882-9914. doi:10.18632/aging.103253

Li, Y., Dammer, E. B., Zhang-Brotzge, X., Chen, S., Duong, D. M., Seyfried, N. T., . . . Sun, Y. Y. (2017). Osteopontin Is a Blood Biomarker for Microglial Activation and Brain Injury in Experimental Hypoxic-Ischemic Encephalopathy. eNeuro, 4(1). doi:10.1523/ENEURO.0253-16.2016

Lindstrom, K., Lagerroos, P., Gillberg, C., & Fernell, E. (2006). Teenage outcome after being born at term with moderate neonatal encephalopathy. Pediatr Neurol, 35(4), 268-274. doi:10.1016/j.pediatrneurol.2006.05.003

Liu, L., Kim, J. Y., Koike, M. A., Yoon, Y. J., Tang, X. N., Ma, H., . . . Yenari, M. A. (2008). FasL shedding is reduced by hypothermia in experimental stroke. J Neurochem, 106(2), 541-550. doi:10.1111/j.1471-4159.2008.05411.x

Liu, X., Wang, M., Chen, H., Guo, Y., Ma, F., Shi, F., . . . Li, Y. (2013). Hypothermia protects the brain from transient global ischemia/reperfusion by attenuating endoplasmic reticulum response-induced apoptosis through CHOP. PLoS One, 8(1), e53431. doi:10.1371/journal.pone.0053431

Liverman, C. S., Kaftan, H. A., Cui, L., Hersperger, S. G., Taboada, E., Klein, R. M., & Berman, N. E. (2006). Altered expression of pro-inflammatory and developmental genes in the fetal brain in a mouse model of maternal infection. Neurosci Lett, 399(3), 220-225. doi:10.1016/j.neulet.2006.01.064

Lobo-Silva, D., Carriche, G. M., Castro, A. G., Roque, S., & Saraiva, M. (2017). Interferon-beta regulates the production of IL-10 by toll-like receptor-activated microglia. Glia, 65(9), 1439-1451. doi:10.1002/glia.23172

Lofqvist, C., Engstrom, E., Sigurdsson, J., Hard, A. L., Niklasson, A., Ewald, U., . . . Hellstrom, A. (2006). Postnatal head growth deficit among premature infants parallels retinopathy of prematurity and insulin-like growth factor-1 deficit. Pediatrics, 117(6), 1930-1938. doi:10.1542/peds.2005-1926

Looney, A. M., Ahearne, C., Boylan, G. B., & Murray, D. M. (2015). Glial Fibrillary Acidic Protein Is Not an Early Marker of Injury in Perinatal Asphyxia and Hypoxic-Ischemic Encephalopathy. Front Neurol, 6, 264. doi:10.3389/fneur.2015.00264

Looney, A. M., Walsh, B. H., Moloney, G., Grenham, S., Fagan, A., O'Keeffe, G. W., . . . Murray, D. M. (2015). Downregulation of Umbilical Cord Blood Levels of miR-374a in Neonatal Hypoxic Ischemic Encephalopathy. J Pediatr, 167(2), 269-273 e262. doi:10.1016/j.jpeds.2015.04.060

Lorek, A., Takei, Y., Cady, E. B., Wyatt, J. S., Penrice, J., Edwards, A. D., . . . 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(6), 699-706. doi:10.1203/00006450-199412000-00003

Lv, H. Y., Wu, S. J., Wang, Q. L., Yang, L. H., Ren, P. S., Qiao, B. J., . . . Li, L. X. (2017). Effect of erythropoietin combined with hypothermia on serum tau protein levels and neurodevelopmental outcome in neonates with hypoxic-ischemic encephalopathy. Neural Regen Res, 12(10), 1655-1663. doi:10.4103/1673-5374.217338

Ly, A., Nikolaev, A., Suresh, G., Zheng, Y., Tessier-Lavigne, M., & Stein, E. (2008). DSCAM is a netrin receptor that collaborates with DCC in mediating turning responses to netrin-1. Cell, 133(7), 1241-1254. doi:10.1016/j.cell.2008.05.030

Ma, Q., & Zhang, L. (2015). Epigenetic programming of hypoxic-ischemic encephalopathy in response to fetal hypoxia. Prog Neurobiol, 124, 28-48. doi:10.1016/j.pneurobio.2014.11.001

Magdalon, J., Mansur, F., Teles, E. S. A. L., de Goes, V. A., Reiner, O., & Sertie, A. L. (2020). Complement System in Brain Architecture and Neurodevelopmental Disorders. Front Neurosci, 14, 23. doi:10.3389/fnins.2020.00023

Malla, R. R., Asimi, R., Teli, M. A., Shaheen, F., & Bhat, M. A. (2017). Erythropoietin monotherapy in perinatal asphyxia with moderate to severe encephalopathy: a randomized placebo-controlled trial. J Perinatol, 37(5), 596-601. doi:10.1038/jp.2017.17

Mallard, C., Ek, C. J., & Vexler, Z. S. (2018). The myth of the immature barrier systems in the developing brain: role in perinatal brain injury. J Physiol, 596(23), 5655-5664. doi:10.1113/JP274938

Mamczur, P., Borsuk, B., Paszko, J., Sas, Z., Mozrzymas, J., Wisniewski, J. R., . . . Rakus, D. (2015). Astrocyte-neuron crosstalk regulates the expression and subcellular localization of carbohydrate metabolism enzymes. Glia, 63(2), 328-340. doi:10.1002/glia.22753

Martinello, K., Hart, A. R., Yap, S., Mitra, S., & Robertson, N. J. (2017). Management and investigation of neonatal encephalopathy: 2017 update. Arch Dis Child Fetal Neonatal Ed, 102(4), F346-F358. doi:10.1136/archdischild-2015-309639

Martinello, K. A., Meehan, C., Avdic-Belltheus, A., Lingam, I., Ragab, S., Hristova, M., . . . Robertson, N. J. (2019). Acute LPS sensitization and continuous infusion exacerbates hypoxic brain injury in a piglet model of neonatal encephalopathy. Sci Rep, 9(1), 10184. doi:10.1038/s41598-019-46488-y

Martinez-Biarge, M., Diez-Sebastian, J., Kapellou, O., Gindner, D., Allsop, J. M., Rutherford, M. A., & Cowan, F. M. (2011). Predicting motor outcome and death in term hypoxic-ischemic encephalopathy. Neurology, 76(24), 2055-2061. doi:10.1212/WNL.0b013e31821f442d

Massaro, A. N., Wu, Y. W., Bammler, T. K., Comstock, B., Mathur, A., McKinstry, R. C., . . . Juul, S. (2018). Plasma Biomarkers of Brain Injury in Neonatal Hypoxic-Ischemic Encephalopathy. J Pediatr, 194, 67-75 e61. doi:10.1016/j.jpeds.2017.10.060

Matsumura, S., Kunori, S., Mabuchi, T., Katano, T., Nakazawa, T., Abe, T., . . . Ito, S. (2010). Impairment of CaMKII activation and attenuation of neuropathic pain in mice lacking NR2B phosphorylated at Tyr1472. Eur J Neurosci, 32(5), 798-810. doi:10.1111/j.1460-9568.2010.07348.x

Matthews, L. G., Walsh, B. H., Knutsen, C., Neil, J. J., Smyser, C. D., Rogers, C. E., & Inder, T. E. (2018). Brain growth in the NICU: critical periods of tissue-specific expansion. Pediatr Res, 83(5), 976-981. doi:10.1038/pr.2018.4

McGowan, M. M., O'Kane, A. C., Vezina, G., Chang, T., Bendush, N., Glass, P., . . . Massaro, A. N. (2021). Serial plasma biomarkers of brain injury in infants with neonatal encephalopathy treated with therapeutic hypothermia. Pediatr Res, 90(6), 1228-1234. doi:10.1038/s41390-021-01405-w

McLean, C., & Ferriero, D. (2004). Mechanisms of hypoxic-ischemic injury in the term infant. Semin Perinatol, 28(6), 425-432. doi:10.1053/j.semperi.2004.10.005

McNally, M. A., & Soul, J. S. (2019). Pharmacologic Prevention and Treatment of Neonatal Brain Injury. Clin Perinatol, 46(2), 311-325. doi:10.1016/j.clp.2019.02.006

McQuillen, P. S., Sheldon, R. A., Shatz, C. J., & Ferriero, D. M. (2003). Selective vulnerability of subplate neurons after early neonatal hypoxia-ischemia. J Neurosci, 23(8), 3308-3315. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/12716938

Ment, L. R., Hirtz, D., & Huppi, P. S. (2009). Imaging biomarkers of outcome in the developing preterm brain. Lancet Neurol, 8(11), 1042-1055. doi:10.1016/S1474-4422(09)70257-1

Millar, L. J., Shi, L., Hoerder-Suabedissen, A., & Molnar, Z. (2017). Neonatal Hypoxia Ischaemia: Mechanisms, Models, and Therapeutic Challenges. Front Cell Neurosci, 11, 78. doi:10.3389/fncel.2017.00078

Miller, S. L., Wallace, E. M., & Walker, D. W. (2012). Antioxidant therapies: a potential role in perinatal medicine. Neuroendocrinology, 96(1), 13-23. doi:10.1159/000336378

Miller, S. P., & Ferriero, D. M. (2009). From selective vulnerability to connectivity: insights from newborn brain imaging. Trends Neurosci, 32(9), 496-505. doi:10.1016/j.tins.2009.05.010

Miller, S. P., Ramaswamy, V., Michelson, D., Barkovich, A. J., Holshouser, B., Wycliffe, N., . . . Ferriero, D. M. (2005). Patterns of brain injury in term neonatal encephalopathy. J Pediatr, 146(4), 453-460. doi:10.1016/j.jpeds.2004.12.026

Moon, C. J., Youn, Y. A., Yum, S. K., & Sung, I. K. (2016). Cytokine changes in newborns with therapeutic hypothermia after hypoxic ischemic encephalopathy. J Perinatol, 36(12), 1092-1096. doi:10.1038/jp.2016.132

Moral, Y., Robertson, N. J., Goni-de-Cerio, F., & Alonso-Alconada, D. (2019). [Neonatal hypoxia-ischemia: cellular and molecular brain damage and therapeutic modulation of neurogenesis]. Rev Neurol, 68(1), 23-36. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/30560986

Morales, P., Fiedler, J. L., Andres, S., Berrios, C., Huaiquin, P., Bustamante, D., . . . Herrera-Marschitz, M. (2008). Plasticity of hippocampus following perinatal asphyxia: effects on postnatal apoptosis and neurogenesis. J Neurosci Res, 86(12), 2650-2662. doi:10.1002/jnr.21715

Mukerji, S. S., Katsman, E. A., Wilber, C., Haner, N. A., Selman, W. R., & Hall, A. K. (2007). Activin is a neuronal survival factor that is rapidly increased after transient cerebral ischemia and hypoxia in mice. J Cereb Blood Flow Metab, 27(6), 1161-1172. doi:10.1038/sj.jcbfm.9600423

Mulder, H., Pitchford, N. J., & Marlow, N. (2011). Inattentive behaviour is associated with poor working memory and slow processing speed in very pre-term children in middle childhood. Br J Educ Psychol, 81(Pt 1), 147-160. doi:10.1348/000709910X505527

Mulkey, S. B., Ramakrishnaiah, R. H., McKinstry, R. C., Chang, T., Mathur, A. M., Mayock, D. E., . . . Wu, Y. W. (2017). Erythropoietin and Brain Magnetic Resonance Imaging Findings in Hypoxic-Ischemic Encephalopathy: Volume of Acute Brain Injury and 1-Year Neurodevelopmental Outcome. J Pediatr, 186, 196-199. doi:10.1016/j.jpeds.2017.03.053

Murray, D. M., O'Connor, C. M., Ryan, C. A., Korotchikova, I., & Boylan, G. B. (2016). Early EEG Grade and Outcome at 5 Years After Mild Neonatal Hypoxic Ischemic Encephalopathy. Pediatrics, 138(4). doi:10.1542/peds.2016-0659

Nair, J., & Kumar, V. H. S. (2018). Current and Emerging Therapies in the Management of Hypoxic Ischemic Encephalopathy in Neonates. Children (Basel), 5(7). doi:10.3390/children5070099

Nash, A., Aumann, T. D., Pigoni, M., Lichtenthaler, S. F., Takeshima, H., Munro, K. M., & Gunnersen, J. M. (2020). Lack of Sez6 Family Proteins Impairs Motor Functions, Short-Term Memory, and Cognitive Flexibility and Alters Dendritic Spine Properties. Cerebral Cortex, 30(4), 2167-2184. doi:10.1093/cercor/bhz230

Ng, P. C., & Lam, H. S. (2012). Biomarkers in neonatology: the next generation of tests. Neonatology, 102(2), 145-151. doi:10.1159/000338587

Norman, M., Hallberg, B., Abrahamsson, T., Bjorklund, L. J., Domellof, M., Farooqi, A., . . . Hakansson, S. (2019). Association Between Year of Birth and 1-Year Survival Among Extremely Preterm Infants in Sweden During 2004-2007 and 2014-2016. JAMA, 321(12), 1188-1199. doi:10.1001/jama.2019.2021

O'Shea, T. M., Joseph, R. M., Kuban, K. C., Allred, E. N., Ware, J., Coster, T., . . . Investigators, E. S. (2014). Elevated blood levels of inflammation-related proteins are associated with an attention problem at age 24 mo in extremely preterm infants. Pediatr Res, 75(6), 781-787. doi:10.1038/pr.2014.41

Ohlsson, A., & Aher, S. M. (2020). Early erythropoiesis-stimulating agents in preterm or low birth weight infants. Cochrane Database Syst Rev, 2, CD004863. doi:10.1002/14651858.CD004863.pub6

Ohno, Y., & Tokudome, K. (2017). Therapeutic Role of Synaptic Vesicle Glycoprotein 2A (SV2A) in Modulating Epileptogenesis. CNS Neurol Disord Drug Targets, 16(4), 463-471. doi:10.2174/1871527316666170404115027

Olsson, A., Kayhan, G., Lagercrantz, H., & Herlenius, E. (2003). IL-1 beta depresses respiration and anoxic survival via a prostaglandin-dependent pathway in neonatal rats. Pediatr Res, 54(3), 326-331. doi:10.1203/01.PDR.0000076665.62641.A2

Oorschot, D. E., Sizemore, R. J., & Amer, A. R. (2020). Treatment of Neonatal Hypoxic-Ischemic Encephalopathy with Erythropoietin Alone, and Erythropoietin Combined with Hypothermia: History, Current Status, and Future Research. Int J Mol Sci, 21(4). doi:10.3390/ijms21041487

Ophelders, D., Gussenhoven, R., Klein, L., Jellema, R. K., Westerlaken, R. J. J., Hutten, M. C., . . . Wolfs, T. (2020). Preterm Brain Injury, Antenatal Triggers, and Therapeutics: Timing Is Key. Cells, 9(8). doi:10.3390/cells9081871

Orrock, J. E., Panchapakesan, K., Vezina, G., Chang, T., Harris, K., Wang, Y., . . . Massaro, A. N. (2016). Association of brain injury and neonatal cytokine response during therapeutic hypothermia in newborns with hypoxic-ischemic encephalopathy. Pediatr Res, 79(5), 742-747. doi:10.1038/pr.2015.280

Ottens, A. K., Bustamante, L., Golden, E. C., Yao, C., Hayes, R. L., Wang, K. K., . . . Dave, J. R. (2010). Neuroproteomics: a biochemical means to discriminate the extent and modality of brain injury. J Neurotrauma, 27(10), 1837-1852. doi:10.1089/neu.2010.1374

Oza, S., Lawn, J. E., Hogan, D. R., Mathers, C., & Cousens, S. N. (2015). Neonatal cause-of-death estimates for the early and late neonatal periods for 194 countries: 2000-2013. Bull World Health Organ, 93(1), 19-28. doi:10.2471/BLT.14.139790

Papa, L., Mittal, M. K., Ramirez, J., Silvestri, S., Giordano, P., Braga, C. F., . . . Zonfrillo, M. R. (2017). Neuronal Biomarker Ubiquitin C-Terminal Hydrolase Detects Traumatic Intracranial Lesions on Computed Tomography in Children and Youth with Mild Traumatic Brain Injury. J Neurotrauma, 34(13), 2132-2140. doi:10.1089/neu.2016.4806

Park, W. S., Sung, S. I., Ahn, S. Y., Yoo, H. S., Sung, D. K., Im, G. H., . . . Chang, Y. S. (2015). Hypothermia augments neuroprotective activity of mesenchymal stem cells for neonatal hypoxic-ischemic encephalopathy. PLoS One, 10(3), e0120893. doi:10.1371/journal.pone.0120893

Pataky, R., Howie, F. A., Girardi, G., & Boardman, J. P. (2017). Complement C5a is present in CSF of human newborns and is elevated in association with preterm birth. J Matern Fetal Neonatal Med, 30(20), 2413-2416. doi:10.1080/14767058.2016.1251896

Pek, J. H., Gan, M. Y., Yap, B. J., Seethor, S. T. T., Greenberg, R. G., Hornik, C. P. V., . . . Chong, S. L. (2020). Contemporary trends in global mortality of sepsis among young infants less than 90 days old: protocol for a systematic review and meta-analysis. BMJ Open, 10(7), e038815. doi:10.1136/bmjopen-2020-038815

Pek, J. H., Yap, B. J., Gan, M. Y., Seethor, S. T. T., Greenberg, R., Hornik, C. P. V., . . . Chong, S. L. (2020). Neurocognitive impairment after neonatal sepsis: protocol for a systematic review and meta-analysis. BMJ Open, 10(6), e038816. doi:10.1136/bmjopen-2020-038816

Perin, J., Mulick, A., Yeung, D., Villavicencio, F., Lopez, G., Strong, K. L., . . . Liu, L. (2022). Global, regional, and national causes of under-5 mortality in 2000-19: an updated systematic analysis with implications for the Sustainable Development Goals. Lancet Child Adolesc Health, 6(2), 106-115. doi:10.1016/S2352-4642(21)00311-4

Poppe, T., Thompson, B., Boardman, J. P., Bastin, M. E., Alsweiler, J., Deib, G., . . . Mag, N. U. M. S. G. (2020). Effect of antenatal magnesium sulphate on MRI biomarkers of white matter development at term equivalent age: The magnum study. EBioMedicine, 59, 102957. doi:10.1016/j.ebiom.2020.102957

Practice, A. C. o. O. (2006). ACOG Committee Opinion No. 348, November 2006: Umbilical cord blood gas and acid-base analysis. Obstet Gynecol, 108(5), 1319-1322. doi:10.1097/00006250-200611000-00058

R.D.C. Team. (2010). R: A language and environment for statistical computing. Retrieved from https://www.gbif.org/tool/81287/r-a-language-and-environment-for-statistical-computing

Rao, R., Trivedi, S., Vesoulis, Z., Liao, S. M., Smyser, C. D., & Mathur, A. M. (2017). Safety and Short-Term Outcomes of Therapeutic Hypothermia in Preterm Neonates 34-35 Weeks Gestational Age with Hypoxic-Ischemic Encephalopathy. J Pediatr, 183, 37-42. doi:10.1016/j.jpeds.2016.11.019

Razak, A., & Hussain, A. (2019). Erythropoietin in perinatal hypoxic-ischemic encephalopathy: a systematic review and meta-analysis. J Perinat Med, 47(4), 478-489. doi:10.1515/jpm-2018-0360

Reid, G., Kirschner, M. B., & van Zandwijk, N. (2011). Circulating microRNAs: Association with disease and potential use as biomarkers. Crit Rev Oncol Hematol, 80(2), 193-208. doi:10.1016/j.critrevonc.2010.11.004

Robertson, N. J., Lingam, I., Meehan, C., Martinello, K. A., Avdic-Belltheus, A., Stein, L., . . . Golay, X. (2020). High-Dose Melatonin and Ethanol Excipient Combined with Therapeutic Hypothermia in a Newborn Piglet Asphyxia Model. Sci Rep, 10(1), 3898. doi:10.1038/s41598-020-60858-x

Robertson, N. J., Meehan, C., Martinello, K. A., Avdic-Belltheus, A., Boggini, T., Mutshiya, T., . . . Lowdell, M. W. (2021). Human umbilical cord mesenchymal stromal cells as an adjunct therapy with therapeutic hypothermia in a piglet model of perinatal asphyxia. Cytotherapy, 23(6), 521-535. doi:10.1016/j.jcyt.2020.10.005

Rocha-Ferreira, E., & Hristova, M. (2015). Antimicrobial peptides and complement in neonatal hypoxia-ischemia induced brain damage. Front Immunol, 6, 56. doi:10.3389/fimmu.2015.00056

Rocha-Ferreira, E., Kelen, D., Faulkner, S., Broad, K. D., Chandrasekaran, M., Kerenyi, A., . . . Robertson, N. J. (2017). Systemic pro-inflammatory cytokine status following therapeutic hypothermia in a piglet hypoxia-ischemia model. J Neuroinflammation, 14(1), 44. doi:10.1186/s12974-017-0821-x

Rocha-Ferreira, E., Poupon, L., Zelco, A., Leverin, A. L., Nair, S., Jonsdotter, A., . . . Rahim, A. A. (2018). Neuroprotective exendin-4 enhances hypothermia therapy in a model of hypoxic-ischaemic encephalopathy. Brain, 141(10), 2925-2942. doi:10.1093/brain/awy220

Rocha-Ferreira, E., Sisa, C., Bright, S., Fautz, T., Harris, M., Contreras Riquelme, I., . . . Hristova, M. (2019). Curcumin: Novel Treatment in Neonatal Hypoxic-Ischemic Brain Injury. Front Physiol, 10, 1351. doi:10.3389/fphys.2019.01351

Roka, A., Beko, G., Halasz, J., Toldi, G., Lakatos, P., Azzopardi, D., . . . Szabo, M. (2013). Changes in serum cytokine and cortisol levels in normothermic and hypothermic term neonates after perinatal asphyxia. Inflamm Res, 62(1), 81-87. doi:10.1007/s00011-012-0554-3

Roka, A., Kelen, D., Halasz, J., Beko, G., Azzopardi, D., & Szabo, M. (2012). Serum S100B and neuron-specific enolase levels in normothermic and hypothermic infants after perinatal asphyxia. Acta Paediatr, 101(3), 319-323. doi:10.1111/j.1651-2227.2011.02480.x

Rousset, C. I., Kassem, J., Olivier, P., Chalon, S., Gressens, P., & Saliba, E. (2008). Antenatal bacterial endotoxin sensitizes the immature rat brain to postnatal excitotoxic injury. J Neuropathol Exp Neurol, 67(10), 994-1000. doi:10.1097/NEN.0b013e31818894a1

Rubenstein, J. L. (2011). Annual Research Review: Development of the cerebral cortex: implications for neurodevelopmental disorders. J Child Psychol Psychiatry, 52(4), 339-355. doi:10.1111/j.1469-7610.2010.02307.x

Ruegger, C. M., Davis, P. G., & Cheong, J. L. (2018). Xenon as an adjuvant to therapeutic hypothermia in near-term and term newborns with hypoxic-ischaemic encephalopathy. Cochrane Database Syst Rev, 8, CD012753. doi:10.1002/14651858.CD012753.pub2

Rummel, C., Matsumura, K., & Luheshi, G. N. (2011). Circulating IL-6 contributes to peripheral LPS-induced mPGES-1 expression in the rat brain. Brain Res Bull, 86(5-6), 319-325. doi:10.1016/j.brainresbull.2011.09.006

Russo, V. C., Gluckman, P. D., Feldman, E. L., & Werther, G. A. (2005). The insulin-like growth factor system and its pleiotropic functions in brain. Endocr Rev, 26(7), 916-943. doi:10.1210/er.2004-0024

Sabir, H., Bonifacio, S. L., Gunn, A. J., Thoresen, M., Chalak, L. F., Newborn Brain Society, G., & Publications, C. (2021). Unanswered questions regarding therapeutic hypothermia for neonates with neonatal encephalopathy. Semin Fetal Neonatal Med, 26(5), 101257. doi:10.1016/j.siny.2021.101257

Safety, W. H. O. I. P. o. C. (2001). Biomarkers in risk assessment : validity and validation. World Health Organization.

Sankowski, R., Mader, S., & Valdes-Ferrer, S. I. (2015). Systemic inflammation and the brain: novel roles of genetic, molecular, and environmental cues as drivers of neurodegeneration. Front Cell Neurosci, 9, 28. doi:10.3389/fncel.2015.00028

Sargent, D., Betemps, D., Drouyer, M., Verchere, J., Gaillard, D., Arsac, J. N., . . . Baron, T. (2018). Investigating the neuroprotective effect of AAV-mediated beta-synuclein overexpression in a transgenic model of synucleinopathy. Sci Rep, 8(1), 17563. doi:10.1038/s41598-018-35825-2

Sarnat, H. B., & Sarnat, M. S. (1976). Neonatal encephalopathy following fetal distress. A clinical and electroencephalographic study. Arch Neurol, 33(10), 696-705. doi:10.1001/archneur.1976.00500100030012

Schlapbach, L. J., Aebischer, M., Adams, M., Natalucci, G., Bonhoeffer, J., Latzin, P., . . . Follow-Up, G. (2011). Impact of sepsis on neurodevelopmental outcome in a Swiss National Cohort of extremely premature infants. Pediatrics, 128(2), e348-357. doi:10.1542/peds.2010-3338

Schmitz, T., Felderhoff-Mueser, U., Sifringer, M., Groenendaal, F., Kampmann, S., & Heep, A. (2011). Expression of soluble Fas in the cerebrospinal fluid of preterm infants with posthemorrhagic hydrocephalus and cystic white matter damage. J Perinat Med, 39(1), 83-88. doi:10.1515/jpm.2010.125

Schwartz, N. B., & Domowicz, M. S. (2018). Proteoglycans in brain development and pathogenesis. FEBS Lett, 592(23), 3791-3805. doi:10.1002/1873-3468.13026

Schwenk, J. M., Gry, M., Rimini, R., Uhlen, M., & Nilsson, P. (2008). Antibody suspension bead arrays within serum proteomics. J Proteome Res, 7(8), 3168-3179. doi:10.1021/pr700890b

Sellier, E., Platt, M. J., Andersen, G. L., Krageloh-Mann, I., De La Cruz, J., Cans, C., & Surveillance of Cerebral Palsy, N. (2016). Decreasing prevalence in cerebral palsy: a multi-site European population-based study, 1980 to 2003. Dev Med Child Neurol, 58(1), 85-92. doi:10.1111/dmcn.12865

Serajee, F. J., & Huq, A. M. (2015). Homozygous Mutation in Synaptic Vesicle Glycoprotein 2A Gene Results in Intractable Epilepsy, Involuntary Movements, Microcephaly, and Developmental and Growth Retardation. Pediatr Neurol, 52(6), 642-646 e641. doi:10.1016/j.pediatrneurol.2015.02.011

Serenius, F., Ewald, U., Farooqi, A., Fellman, V., Hafstrom, M., Hellgren, K., . . . Extremely Preterm Infants in Sweden Study, G. (2016). Neurodevelopmental Outcomes Among Extremely Preterm Infants 6.5 Years After Active Perinatal Care in Sweden. JAMA Pediatr, 170(10), 954-963. doi:10.1001/jamapediatrics.2016.1210

Sfaello, I., Baud, O., Arzimanoglou, A., & Gressens, P. (2005). Topiramate prevents excitotoxic damage in the newborn rodent brain. Neurobiol Dis, 20(3), 837-848. doi:10.1016/j.nbd.2005.05.019

Shah, P. S. (2010). Hypothermia: a systematic review and meta-analysis of clinical trials. Semin Fetal Neonatal Med, 15(5), 238-246. doi:10.1016/j.siny.2010.02.003

Shankaran, S. (2012a). Hypoxic-ischemic encephalopathy and novel strategies for neuroprotection. Clin Perinatol, 39(4), 919-929. doi:10.1016/j.clp.2012.09.008

Shankaran, S. (2012b). Therapeutic hypothermia for neonatal encephalopathy. Curr Treat Options Neurol, 14(6), 608-619. doi:10.1007/s11940-012-0200-y

Shankaran, S., Laptook, A. R., Pappas, A., McDonald, S. A., Das, A., Tyson, J. E., . . . Human Development Neonatal Research, N. (2014). Effect of depth and duration of cooling on deaths in the NICU among neonates with hypoxic ischemic encephalopathy: a randomized clinical trial. JAMA, 312(24), 2629-2639. doi:10.1001/jama.2014.16058

Shankaran, S., Laptook, A. R., Pappas, A., McDonald, S. A., Das, A., Tyson, J. E., . . . Human Development Neonatal Research, N. (2017). Effect of Depth and Duration of Cooling on Death or Disability at Age 18 Months Among Neonates With Hypoxic-Ischemic Encephalopathy: A Randomized Clinical Trial. JAMA, 318(1), 57-67. doi:10.1001/jama.2017.7218

Shankaran, S., Laptook, A. R., Poole, W. K., & Eunice Kennedy Shriver, N. N. R. N. (2010). Hypothermia for perinatal asphyxial encephalopathy. N Engl J Med, 362(11), 1051-1052; author reply 1052. doi:10.1056/NEJMc0912848

Sheldon, R. A., Windsor, C., Lee, B. S., Arteaga Cabeza, O., & Ferriero, D. M. (2017). Erythropoietin Treatment Exacerbates Moderate Injury after Hypoxia-Ischemia in Neonatal Superoxide Dismutase Transgenic Mice. Dev Neurosci, 39(1-4), 228-237. doi:10.1159/000472710

Siljehav, V., Hofstetter, A. M., Leifsdottir, K., & Herlenius, E. (2015). Prostaglandin E2 Mediates Cardiorespiratory Disturbances during Infection in Neonates. J Pediatr, 167(6), 1207-1213 e1203. doi:10.1016/j.jpeds.2015.08.053

Siljehav, V., Olsson Hofstetter, A., Jakobsson, P. J., & Herlenius, E. (2012). mPGES-1 and prostaglandin E2: vital role in inflammation, hypoxic response, and survival. Pediatr Res, 72(5), 460-467. doi:10.1038/pr.2012.119

Simola, N., Bustamante, D., Pinna, A., Pontis, S., Morales, P., Morelli, M., & Herrera-Marschitz, M. (2008). Acute perinatal asphyxia impairs non-spatial memory and alters motor coordination in adult male rats. Exp Brain Res, 185(4), 595-601. doi:10.1007/s00221-007-1186-7

Smith, P. L., Hagberg, H., Naylor, A. S., & Mallard, C. (2014). Neonatal peripheral immune challenge activates microglia and inhibits neurogenesis in the developing murine hippocampus. Dev Neurosci, 36(2), 119-131. doi:10.1159/000359950

Sofroniew, M. V., & Vinters, H. V. (2010). Astrocytes: biology and pathology. Acta Neuropathol, 119(1), 7-35. doi:10.1007/s00401-009-0619-8

Song, J., Sun, H., Xu, F., Kang, W., Gao, L., Guo, J., . . . Zhu, C. (2016). Recombinant human erythropoietin improves neurological outcomes in very preterm infants. Ann Neurol, 80(1), 24-34. doi:10.1002/ana.24677

Soul, J. S., & Spence, S. J. (2020). Predicting Autism Spectrum Disorder in Very Preterm Infants. Pediatrics, 146(4). doi:10.1542/peds.2020-019448

Spittle, A. J., Cameron, K., Doyle, L. W., Cheong, J. L., & Victorian Infant Collaborative Study, G. (2018). Motor Impairment Trends in Extremely Preterm Children: 1991-2005. Pediatrics, 141(4). doi:10.1542/peds.2017-3410

Steinman, K. J., Gorno-Tempini, M. L., Glidden, D. V., Kramer, J. H., Miller, S. P., Barkovich, A. J., & Ferriero, D. M. (2009). Neonatal watershed brain injury on magnetic resonance imaging correlates with verbal IQ at 4 years. Pediatrics, 123(3), 1025-1030. doi:10.1542/peds.2008-1203

Stoll, B. J., Hansen, N. I., Bell, E. F., Walsh, M. C., Carlo, W. A., Shankaran, S., . . . Human Development Neonatal Research, N. (2015). Trends in Care Practices, Morbidity, and Mortality of Extremely Preterm Neonates, 1993-2012. JAMA, 314(10), 1039-1051. doi:10.1001/jama.2015.10244

Stoll, G., Jander, S., & Schroeter, M. (1998). Inflammation and glial responses in ischemic brain lesions. Prog Neurobiol, 56(2), 149-171. doi:10.1016/s0301-0082(98)00034-3

Sugiyama, Y., Oishi, T., Yamashita, A., Murata, Y., Yamamoto, T., Takashima, I., . . . Higo, N. (2019). Neuronal and microglial localization of secreted phosphoprotein 1 (osteopontin) in intact and damaged motor cortex of macaques. Brain Res, 1714, 52-64. doi:10.1016/j.brainres.2019.02.021

Sullivan, B. A., Grice, S. M., Lake, D. E., Moorman, J. R., & Fairchild, K. D. (2014). Infection and other clinical correlates of abnormal heart rate characteristics in preterm infants. J Pediatr, 164(4), 775-780. doi:10.1016/j.jpeds.2013.11.038

Sumanovic-Glamuzina, D., Culo, F., Culo, M. I., Konjevoda, P., Bozic, T., Robovic, A., . . . Kuzman, Z. (2008). Vasodilatory prostaglandins in perinatal hypoxic brain damage. Coll Antropol, 32 Suppl 1, 183-187. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/18405080

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(3), 464-479. doi:10.1038/jcbfm.2008.141

Tagin, M., Shah, P. S., & Lee, K. S. (2013). Magnesium for newborns with hypoxic-ischemic encephalopathy: a systematic review and meta-analysis. J Perinatol, 33(9), 663-669. doi:10.1038/jp.2013.65

Tagin, M. A., Woolcott, C. G., Vincer, M. J., Whyte, R. K., & Stinson, D. A. (2012). Hypothermia for neonatal hypoxic ischemic encephalopathy: an updated systematic review and meta-analysis. Arch Pediatr Adolesc Med, 166(6), 558-566. doi:10.1001/archpediatrics.2011.1772

Takahashi, K., Hasegawa, S., Maeba, S., Fukunaga, S., Motoyama, M., Hamano, H., & Ichiyama, T. (2014). Serum tau protein level serves as a predictive factor for neurological prognosis in neonatal asphyxia. Brain Dev, 36(8), 670-675. doi:10.1016/j.braindev.2013.10.007

Tan, M. G., Lee, C., Lee, J. H., Francis, P. T., Williams, R. J., Ramirez, M. J., . . . Lai, M. K. (2014). Decreased rabphilin 3A immunoreactivity in Alzheimer's disease is associated with Abeta burden. Neurochem Int, 64, 29-36. doi:10.1016/j.neuint.2013.10.013

Tetorou, K., Sisa, C., Iqbal, A., Dhillon, K., & Hristova, M. (2021). Current Therapies for Neonatal Hypoxic-Ischaemic and Infection-Sensitised Hypoxic-Ischaemic Brain Damage. Front Synaptic Neurosci, 13, 709301. doi:10.3389/fnsyn.2021.709301

Thatipamula, S., & Hossain, M. A. (2014). Critical role of extracellularly secreted neuronal pentraxin 1 in ischemic neuronal death. BMC Neurosci, 15, 133. doi:10.1186/s12868-014-0133-3

Thelin, E. P., Jeppsson, E., Frostell, A., Svensson, M., Mondello, S., Bellander, B. M., & Nelson, D. W. (2016). Utility of neuron-specific enolase in traumatic brain injury; relations to S100B levels, outcome, and extracranial injury severity. Crit Care, 20, 285. doi:10.1186/s13054-016-1450-y

Thelin, E. P., Just, D., Frostell, A., Haggmark-Manberg, A., Risling, M., Svensson, M., . . . Bellander, B. M. (2018). Protein profiling in serum after traumatic brain injury in rats reveals potential injury markers. Behav Brain Res, 340, 71-80. doi:10.1016/j.bbr.2016.08.058

Thompson, D. K., Kelly, C. E., Chen, J., Beare, R., Alexander, B., Seal, M. L., . . . Spittle, A. J. (2019). Characterisation of brain volume and microstructure at term-equivalent age in infants born across the gestational age spectrum. Neuroimage Clin, 21, 101630. doi:10.1016/j.nicl.2018.101630

Thompson, D. K., Loh, W. Y., Connelly, A., Cheong, J. L. Y., Spittle, A. J., Chen, J., . . . Anderson, P. J. (2020). Basal ganglia and thalamic tract connectivity in very preterm and full-term children; associations with 7-year neurodevelopment. Pediatr Res, 87(1), 48-56. doi:10.1038/s41390-019-0546-x

Thoresen, M., Liu, X., Jary, S., Brown, E., Sabir, H., Stone, J., . . . Karlsson, M. (2012). Lactate dehydrogenase in hypothermia-treated newborn infants with hypoxic-ischaemic encephalopathy. Acta Paediatr, 101(10), 1038-1044. doi:10.1111/j.1651-2227.2012.02778.x

Thoresen, M., Tooley, J., Liu, X., Jary, S., Fleming, P., Luyt, K., . . . Sabir, H. (2013). Time is brain: starting therapeutic hypothermia within three hours after birth improves motor outcome in asphyxiated newborns. Neonatology, 104(3), 228-233. doi:10.1159/000353948

Thornton, C., Leaw, B., Mallard, C., Nair, S., Jinnai, M., & Hagberg, H. (2017). Cell Death in the Developing Brain after Hypoxia-Ischemia. Front Cell Neurosci, 11, 248. doi:10.3389/fncel.2017.00248

Thornton, C., Rousset, C. I., Kichev, A., Miyakuni, Y., Vontell, R., Baburamani, A. A., . . . Hagberg, H. (2012). Molecular mechanisms of neonatal brain injury. Neurol Res Int, 2012, 506320. doi:10.1155/2012/506320

Tohyama, J., Nakashima, M., Nabatame, S., Gaik-Siew, C., Miyata, R., Rener-Primec, Z., . . . Saitsu, H. (2015). SPTAN1 encephalopathy: distinct phenotypes and genotypes. J Hum Genet, 60(4), 167-173. doi:10.1038/jhg.2015.5

Tomlinson, M. S., Santos, H. P., Stewart, J. R., Joseph, R., Leviton, A., Onderdonk, A. B., . . . Investigators, E. S. (2020). Neurocognitive and social-communicative function of children born very preterm at 10 years of age: Associations with microorganisms recovered from the placenta parenchyma. J Perinatol, 40(2), 306-315. doi:10.1038/s41372-019-0505-8

Toorell, H., Zetterberg, H., Blennow, K., Savman, K., & Hagberg, H. (2018). Increase of neuronal injury markers Tau and neurofilament light proteins in umbilical blood after intrapartum asphyxia. J Matern Fetal Neonatal Med, 31(18), 2468-2472. doi:10.1080/14767058.2017.1344964

Tran, K. D., Cernichiaro-Espinosa, L. A., & Berrocal, A. M. (2018). Management of Retinopathy of Prematurity--Use of Anti-VEGF Therapy. Asia Pac J Ophthalmol (Phila), 7(1), 56-62. doi:10.22608/APO.2017436

Traudt, C. M., McPherson, R. J., Bauer, L. A., Richards, T. L., Burbacher, T. M., McAdams, R. M., & Juul, S. E. (2013). Concurrent erythropoietin and hypothermia treatment improve outcomes in a term nonhuman primate model of perinatal asphyxia. Dev Neurosci, 35(6), 491-503. doi:10.1159/000355460

Tsuji, M., Wilson, M. A., Lange, M. S., & Johnston, M. V. (2004). Minocycline worsens hypoxic-ischemic brain injury in a neonatal mouse model. Exp Neurol, 189(1), 58-65. doi:10.1016/j.expneurol.2004.01.011

Tsuji, S., Di Martino, E., Mukai, T., Tsuji, S., Murakami, T., Harris, R. A., . . . Aden, U. (2020). Aggravated brain injury after neonatal hypoxic ischemia in microglia-depleted mice. J Neuroinflammation, 17(1), 111. doi:10.1186/s12974-020-01792-7

Turkyilmaz, C., Turkyilmaz, Z., Atalay, Y., Soylemezoglu, F., & Celasun, B. (2002). Magnesium pre-treatment reduces neuronal apoptosis in newborn rats in hypoxia-ischemia. Brain Res, 955(1-2), 133-137. doi:10.1016/s0006-8993(02)03395-4

Tuttolomondo, A., Di Raimondo, D., di Sciacca, R., Pinto, A., & Licata, G. (2008). Inflammatory cytokines in acute ischemic stroke. Curr Pharm Des, 14(33), 3574-3589. doi:10.2174/138161208786848739

Uhlen, M., Oksvold, P., Fagerberg, L., Lundberg, E., Jonasson, K., Forsberg, M., . . . Ponten, F. (2010). Towards a knowledge-based Human Protein Atlas. Nat Biotechnol, 28(12), 1248-1250. doi:10.1038/nbt1210-1248

Ulgen, E., Ozisik, O., & Sezerman, O. U. (2019). pathfindR: An R Package for Comprehensive Identification of Enriched Pathways in Omics Data Through Active Subnetworks. Front Genet, 10, 858. doi:10.3389/fgene.2019.00858

van den Heuij, L. G., Fraser, M., Miller, S. L., Jenkin, G., Wallace, E. M., Davidson, J. O., . . . Bennet, L. (2019). Delayed intranasal infusion of human amnion epithelial cells improves white matter maturation after asphyxia in preterm fetal sheep. J Cereb Blood Flow Metab, 39(2), 223-239. doi:10.1177/0271678X17729954

van Landeghem, F. K., 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(2), 129-135. doi:10.1203/00006450-200202000-00003

van Velthoven, C. T., Kavelaars, A., van Bel, F., & Heijnen, C. J. (2010). Mesenchymal stem cell treatment after neonatal hypoxic-ischemic brain injury improves behavioral outcome and induces neuronal and oligodendrocyte regeneration. Brain Behav Immun, 24(3), 387-393. doi:10.1016/j.bbi.2009.10.017

Vanden Berghe, T., Linkermann, A., Jouan-Lanhouet, S., Walczak, H., & Vandenabeele, P. (2014). Regulated necrosis: the expanding network of non-apoptotic cell death pathways. Nat Rev Mol Cell Biol, 15(2), 135-147. doi:10.1038/nrm3737

VanderVeen, D. K., Melia, M., Yang, M. B., Hutchinson, A. K., Wilson, L. B., & Lambert, S. R. (2017). Anti-Vascular Endothelial Growth Factor Therapy for Primary Treatment of Type 1 Retinopathy of Prematurity: A Report by the American Academy of Ophthalmology. Ophthalmology, 124(5), 619-633. doi:10.1016/j.ophtha.2016.12.025

Vanes, L. D., Murray, R. M., & Nosarti, C. (2021). Adult outcome of preterm birth: Implications for neurodevelopmental theories of psychosis. Schizophr Res. doi:10.1016/j.schres.2021.04.007

Vannucci, R. C., Connor, J. R., Mauger, D. T., Palmer, C., Smith, M. B., Towfighi, J., & Vannucci, S. J. (1999). Rat model of perinatal hypoxic-ischemic brain damage. J Neurosci Res, 55(2), 158-163. doi:10.1002/(SICI)1097-4547(19990115)55:2<158::AID-JNR3>3.0.CO;2-1

Vannucci, S. J., & Hagberg, H. (2004). Hypoxia-ischemia in the immature brain. J Exp Biol, 207(Pt 18), 3149-3154. doi:10.1242/jeb.01064

Vegas, N., Cavallin, M., Kleefstra, T., de Boer, L., Philbert, M., Maillard, C., . . . Bahi-Buisson, N. (2018). Mutations in TBR1 gene leads to cortical malformations and intellectual disability. Eur J Med Genet, 61(12), 759-764. doi:10.1016/j.ejmg.2018.09.012

Venkatesh, K. K., Leviton, A., Hecht, J. L., Joseph, R. M., Douglass, L. M., Frazier, J. A., . . . Kuban, K. C. K. (2020). Histologic chorioamnionitis and risk of neurodevelopmental impairment at age 10 years among extremely preterm infants born before 28 weeks of gestation. Am J Obstet Gynecol. doi:10.1016/j.ajog.2020.05.001

Vilanova, J. M., Figueras-Aloy, J., Rosello, J., Gomez, G., Gelpi, E., & Jimenez, R. (1998). Arachidonic acid metabolites in CSF in hypoxic-ischaemic encephalopathy of newborn infants. Acta Paediatr, 87(5), 588-592. doi:10.1080/08035259850158344

Vincer, M. J., Allen, A. C., Allen, V. M., Baskett, T. F., & O'Connell, C. M. (2014). Trends in the prevalence of cerebral palsy among very preterm infants (<31 weeks' gestational age). Paediatr Child Health, 19(4), 185-189. doi:10.1093/pch/19.4.185

Volpe, J. J. (2009). Brain injury in premature infants: a complex amalgam of destructive and developmental disturbances. Lancet Neurol, 8(1), 110-124. doi:10.1016/S1474-4422(08)70294-1

Volpe, J. J. (2019a). Dysmaturation of Premature Brain: Importance, Cellular Mechanisms, and Potential Interventions. Pediatr Neurol, 95, 42-66. doi:10.1016/j.pediatrneurol.2019.02.016

Volpe, J. J. (2019b). Microglia: Newly discovered complexity could lead to targeted therapy for neonatal white matter injury and dysmaturation. J Neonatal Perinatal Med, 12(3), 239-242. doi:10.3233/NPM-190303

Volpe, J. J. (2019c). Placental assessment provides insight into mechanisms and timing of neonatal hypoxic-ischemic encephalopathy. J Neonatal Perinatal Med, 12(2), 113-116. doi:10.3233/NPM-190270

Walsh, B. H., Boylan, G. B., Dempsey, E. M., & Murray, D. M. (2013). Association of nucleated red blood cells and severity of encephalopathy in normothermic and hypothermic infants. Acta Paediatr, 102(2), e64-67. doi:10.1111/apa.12086

Walsh, B. H., Boylan, G. B., & Murray, D. M. (2011). Nucleated red blood cells and early EEG: predicting Sarnat stage and two year outcome. Early Hum Dev, 87(5), 335-339. doi:10.1016/j.earlhumdev.2011.01.041

Wassink, G., Davidson, J. O., Dhillon, S. K., Zhou, K., Bennet, L., Thoresen, M., & Gunn, A. J. (2019). Therapeutic Hypothermia in Neonatal Hypoxic-Ischemic Encephalopathy. Curr Neurol Neurosci Rep, 19(2), 2. doi:10.1007/s11910-019-0916-0

Wassink, G., Davidson, J. O., Fraser, M., Yuill, C. A., Bennet, L., & Gunn, A. J. (2020). Non-additive effects of adjunct erythropoietin therapy with therapeutic hypothermia after global cerebral ischaemia in near-term fetal sheep. J Physiol, 598(5), 999-1015. doi:10.1113/JP279131

Wassink, G., Gunn, E. R., Drury, P. P., Bennet, L., & Gunn, A. J. (2014). The mechanisms and treatment of asphyxial encephalopathy. Front Neurosci, 8, 40. doi:10.3389/fnins.2014.00040

Weston, E. J., Pondo, T., Lewis, M. M., Martell-Cleary, P., Morin, C., Jewell, B., . . . Schrag, S. J. (2011). The burden of invasive early-onset neonatal sepsis in the United States, 2005-2008. Pediatr Infect Dis J, 30(11), 937-941. doi:10.1097/INF.0b013e318223bad2

Whitelaw, A., Rosengren, L., & Blennow, M. (2001). Brain specific proteins in posthaemorrhagic ventricular dilatation. Arch Dis Child Fetal Neonatal Ed, 84(2), F90-91. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11207222

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1721221/pdf/v084p00F90.pdf

Wood, T. L., Loladze, V., Altieri, S., Gangoli, N., Levison, S. W., Brywe, K. G., . . . Hagberg, H. (2007). Delayed IGF-1 administration rescues oligodendrocyte progenitors from glutamate-induced cell death and hypoxic-ischemic brain damage. Dev Neurosci, 29(4-5), 302-310. doi:10.1159/000105471

Wu, L., Xiong, X., Wu, X., Ye, Y., Jian, Z., Zhi, Z., & Gu, L. (2020). Targeting Oxidative Stress and Inflammation to Prevent Ischemia-Reperfusion Injury. Front Mol Neurosci, 13, 28. doi:10.3389/fnmol.2020.00028

Wu, Y., Stoodley, C., Brossard-Racine, M., Kapse, K., Vezina, G., Murnick, J., . . . Limperopoulos, C. (2020). Altered local cerebellar and brainstem development in preterm infants. Neuroimage, 213, 116702. doi:10.1016/j.neuroimage.2020.116702

Xu, G., Broadbelt, K. G., Haynes, R. L., Folkerth, R. D., Borenstein, N. S., Belliveau, R. A., . . . Kinney, H. C. (2011). Late development of the GABAergic system in the human cerebral cortex and white matter. J Neuropathol Exp Neurol, 70(10), 841-858. doi:10.1097/NEN.0b013e31822f471c

Yang, L., Yu, X., Zhang, Y., Liu, N., Xue, X., & Fu, J. (2021). Encephalopathy in Preterm Infants: Advances in Neuroprotection With Caffeine. Front Pediatr, 9, 724161. doi:10.3389/fped.2021.724161

Ye, L., Feng, Z., Doycheva, D., Malaguit, J., Dixon, B., Xu, N., . . . Tang, J. (2018). CpG-ODN exerts a neuroprotective effect via the TLR9/pAMPK signaling pathway by activation of autophagy in a neonatal HIE rat model. Exp Neurol, 301(Pt A), 70-80. doi:10.1016/j.expneurol.2017.12.008

Yook, C., Kim, K., Kim, D., Kang, H., Kim, S. G., Kim, E., & Kim, S. Y. (2019). A TBR1-K228E Mutation Induces Tbr1 Upregulation, Altered Cortical Distribution of Interneurons, Increased Inhibitory Synaptic Transmission, and Autistic-Like Behavioral Deficits in Mice. Front Mol Neurosci, 12, 241. doi:10.3389/fnmol.2019.00241

Yum, S. K., Moon, C. J., Youn, Y. A., & Sung, I. K. (2017). Changes in lactate dehydrogenase are associated with central gray matter lesions in newborns with hypoxic-ischemic encephalopathy. J Matern Fetal Neonatal Med, 30(10), 1177-1181. doi:10.1080/14767058.2016.1208745

Zaigham, M., Kallen, K., Marsal, K., & Olofsson, P. (2020). Hypoxia with acidosis in extremely preterm born infants was not associated with an increased risk of death or impaired neurodevelopmental outcome at 6.5 years. Acta Paediatr, 109(1), 85-92. doi:10.1111/apa.14925

Zhang, J., Luo, X., Huang, C., Pei, Z., Xiao, H., Luo, X., . . . Chang, Y. (2020). Erythropoietin prevents LPS-induced preterm birth and increases offspring survival. Am J Reprod Immunol, 84(3), e13283. doi:10.1111/aji.13283

Zhang, Y., Inder, T. E., Neil, J. J., Dierker, D. L., Alexopoulos, D., Anderson, P. J., & Van Essen, D. C. (2015). Cortical structural abnormalities in very preterm children at 7 years of age. Neuroimage, 109, 469-479. doi:10.1016/j.neuroimage.2015.01.005

Zhong, W., Danielsson, H., Tebani, A., Karlsson, M. J., Elfvin, A., Hellgren, G., . . . Uhlen, M. (2021). Dramatic changes in blood protein levels during the first week of life in extremely preterm infants. Pediatr Res, 89(3), 604-612. doi:10.1038/s41390-020-0912-8

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REGULAR ARTICLE

PGE_2 – metabolite levels in CSF correlate to HIE score and outcome after perinatal asphyxia

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ABSTRACT

Aim: Acute anoxic exposure rapidly increases prostaglandin E_2 (PGE_2) production and release in neonatal mice brains. We hypothesize that PGE_2 is released in human cerebrospinal fluid (CSF) during perinatal asphyxia and that it might be used as a biomarker for perinatal asphyxia.

Methods: In full-term infants with lumbar puncture performed within 72 h of birth (n = 35), CSF was analysed for prostaglandin E_2 metabolite (PGEM) using an enzyme immunoassay. Term infants with suspected but unverified infections were used as controls (n = 11). Hypoxic–ischaemic encephalopathy (HIE) was classified as mild, moderate or severe (HIE I-III). Neurological assessment of surviving patients was performed at 18 months of age.

Results: Prostaglandin E_2 metabolite levels correlated to a low Apgar score at 5 min ($p < 0.01$) and 10 min ($p < 0.01$), a low pH ($p < 0.001$) and HIE score ($p < 0.05$). The HIE-III cases (n = 7) had significantly higher PGEM levels compared with both controls and the HIE-I group (n = 8). Irrespective of HIE grade, patients with adverse or fatal outcome had higher PGEM values compared with controls and asphyxiated infants with normal outcome ($p < 0.05$).

Conclusion: PGE_2 is released during anoxic events in newborn infants, and PGEM may be useful as a biomarker for estimating degree of insult and predicting long-term outcome after perinatal asphyxia.

INTRODUCTION

Birth asphyxia is a major complication during delivery and remains a main cause for perinatal morbidity and mortality with an incidence of 1.2–7.7 per 1000 births in resource-rich countries (1). Half of these lead to severe asphyxia and life-threatening conditions. In about 25% of cases, ischaemic brain damage causes neurological sequelae or death (1). Hypoxic–ischaemic encephalopathy (HIE) designates the condition of a full-term infant who has experienced a perinatal deficit in cerebral oxygen delivery leading to disruption of cerebral energy metabolism (2).

The local brain ischaemia leads to a hypoxic injury and acute response with immediate release of neuromodulators, for example, adenosine, and rapid induction of a number of pro-inflammatory mediators. After a primary disruption of oxygen delivery to the brain, there is a secondary phase of neuronal loss, which can be initiated hours or days later (3). This delayed response may partially be due to secondary induced inflammatory mediators that are released in response to the hypoxic injury (4). Such mediators include prostaglandins, especially PGE_2, which is involved in the secondary phase as well as in the immediate response to acute hypoxia.

Acute hypoxia increases the activity of mPGES-1 (microsomal prostaglandin E synthase–1) in endothelial cells of the blood–brain barrier (BBB) and the subsequent release of prostaglandin E_2 (PGE_2) beyond the BBB (5–7). Also in *ex vivo* experiments, anoxia increases the PGE_2 production in mouse cortex (8) and transient asphyxia elevates the PGE_2 concentrations in newborn guinea pig brain (9). Moreover, we have shown that transient anoxia induces mPGES-1 activity in the mouse brainstem and that IL-1β further increases the anoxic induction of mPGES-1 activity (7,10).

Key notes

- A rapid release of PGE_2 occurs in newborn infants in response to acute hypoxia and is involved in the immediate response to perinatal asphyxia.
- PGE_2 metabolite levels in CSF correlate to 5 and 10 minute Apgar score, arterial pH and base excess and are a valid discriminator for the degree of HIE and final outcome.
- The PGE_2 metabolite is a biomarker for severity and hypoxic damage in perinatal asphyxia in human infants.

High levels of PGE_2 are detected in all term and preterm newborns, compared with older infants (11). This is particularly true during the third stage of labour where PGE_2 levels seem to peak (12). In the pregnant mother, prostaglandins regulate parturition and in the foetus prostaglandins inhibit breathing moments and maintain patency of the ductus arteriosus to facilitate foetal circulation (13). Prostaglandins exert inhibition of the foetal movements, decrease metabolic rate and energy turnover and thus may protect the brain when oxygen and energy resources might be scarce (14). Therefore, PGE_2 is a vital neuro- and vasomodulator in the foetus, during parturition and in the newborn. PGE_2 is a multifaceted molecular messenger, and depending on what type of receptor it binds to, it can have stimulatory or inhibitory effects (15). PGE_2 acts on four G-protein-coupled receptors (EP1, EP2, EP3 and EP4). EP2, EP3 and EP4 receptors act on adenylate cyclase and may have neuroprotective effects (16). EP1 receptors contribute to neurotoxicity by disrupting Ca^{2+} homeostasis through impairing Na^{+}-Ca^{2+} exchange augmenting the Ca^{2+} dysregulation underlying excitotoxic neuronal death (17).

Thus, PGE_2 levels are increased at birth and possibly even more so during acute hypoxia and can have both neuroprotective and neurotoxic effects. Could it then be used as a biomarker for estimating degree of insult and predicting long-term outcome in asphyxiated newborn children?

Prostaglandin levels in ischaemic brain injury have previously been studied in relation to severity of ischaemia (11,18). However, the level of PGE_2 has primarily been examined in its bioactive nonmetabolite form, which has a short half-life in most biological fluids $t1/2 < 5$ min in plasma (18,19). PGE_2 is primarily catabolized enzymatically by stepwise oxidation and reduction into a metabolite 13,14-dihydro-15-keto PGE_2, which accumulates into more stable (t1/2 approximately 45 min) and detectable levels in biological fluids (20). It is then further nonenzymatically metabolized into a 13,14-dihydro-15-keto PGA_2.

The assay employed in this study uses a derivatization of the two 13,14-dihydro-15-keto metabolites to convert them into a single stable form for detection. By analysing a more stable PGE_2 metabolite (PGEM) in cerebrospinal fluid (CSF), we investigate its possible usefulness as a biomarker and its correlation with the arterial pH, base deficit, stages of HIE and final clinical outcome after perinatal asphyxia.

PATIENTS AND METHODS

Patients

Between October 1999 and September 2004, term infants (>37 week gestational age) were enrolled in a study for inflammatory marker analysis during perinatal asphyxia. All were treated at the neonatal intensive care unit (NICU), Karolinska University Hospital in Stockholm, either due to perinatal asphyxia or, in the case of the control group of infants, examined for suspected perinatal CNS infection.

Thirty-five infants including controls had a lumbar puncture (LP) carried out following clinical indications, met the inclusion criteria and had sufficient material available for analysis of PGE_2 metabolite. Of these, 24 were asphyxiated children who fulfilled two or more of the following inclusion criteria:

1. Signs of foetal distress as indicated by cardiotocographic pattern of late decelerations, absent variability or bradycardia, meconium staining of amniotic fluid, scalp pH <7.2 or lactate >4.8 mmol/L.
2. Postnatal stress as indicated by Apgar score <6 at 5 min and need for neonatal resuscitation in the delivery room for >3 min or umbilical arterial/first postnatal blood pH <7.1, BE <−15 (or lactate >4.8 mm/L) in cord blood or venous blood.
3. Neurological signs of encephalopathy within 6 h of birth, such as suppressed or absent reflexes, neuromuscular control and/or altered autonomic function.

Exclusion criteria were congenital malformations, chromosomal abnormalities and encephalopathy unrelated to asphyxia; metabolic diseases and intrauterine/perinatal infections with confirmed meningitis. The control group (n = 11) consisted of infants: (i) indicated for LP due to suspected infection but negative bacterial and viral cultures from blood and CSF, (ii) no leucocytes and normal amounts of proteins in CSF and (iii) no findings suggesting CNS pathology.

Clinical assessment

Acute neurological assessment was made within the first few hours postnatally before enrolling the patient into the study, then at approximately 12, 36 and 72 h after birth and on day seven on patients in the NICU. HIE was classified as mild (HIE-I), moderate (HIE-II) or severe (HIE-III) according to the criteria of Sarnat and Sarnat (21).

Patients received standard treatment, which at the time of recruitment included fluid restriction, inotropic support and mechanical ventilation when needed, as well as medical treatment of seizure activity. All patients were treated under normothermic conditions. Continuous amplitude-integrated EEG was used to assess all suspected HIE cases in the first days of life. In patients with moderate and severe HIE, a CT or MRI scan of the brain was carried out on the third day of life and EEG registration in the first week. Neurological assessment of surviving patients was made at three, six and eighteen months of age. Final outcome for the eighteenth-month assessment was defined using three categories: normal, adverse or death. Adverse outcome was defined as: cerebral palsy, microcephaly (>2 SD below mean head circumference for age and sex), mental retardation (IQ below 70), seizure disorder and neurodevelopment delay (3). The outcome was defined as normal in the absence of these major disabilities.

CSF sampling

Lumbar punctures (LPs) were performed during the first 72 h of life, and in four cases, an additional LP was performed between 28 and 209 h postnatal age. Each

lumbar puncture collected approximately 1–2 mL of CSF. The samples were spun at 3000 rpm at 4 degrees for 10 min and the supernatant stored at −80 °C in aliquots of 0.5 mL until analysed.

PGE_2 metabolite assay

Collected CSF samples were analysed for PGEM using a commercial standardized competition enzyme immunoassay (EIA) protocol (Cayman Chemicals, Ann Arbor, MI, USA). This is a competition assay that converts 13,14-dihydro-15-keto PGA_2 and 13,14-dihydro-15-keto PGE_2 to a single, stable derivative that is quantified. The calculated value represented the concentration of the analyte present in the samples as compared to the standard curve measured. In 13 cases, the CSF sample retrieved was enough for an additional test run at another time point and the mean of the two analyses was used as the calculated value.

Statistical analysis

Clinical data are presented as means and 95% confidence intervals or interquartile ranges for descriptive purposes unless stated otherwise. Parametric clinical data were tested with Student's *t*-test, and Dunnett's test was applied to analyse differences in pH and base excess (BE) levels between patients and controls. Tukey–Kramer HSD test was used to determine the association between PGEM levels and degree of HIE or clinical outcome. The receiver operating characteristic (ROC) curve displays the false-positive rate (1 − specificity) versus the true-positive rate (sensitivity) for different test cut-off values, thus plotting the performance of a diagnostic test. The indicated cut-off criteria were chosen geometrically towards an ideal test with 100% specificity and sensitivity. Data are presented as mean + SEM. p-values of <0.05 were considered statistically significant. Statistical analysis and graphical software utilized were JMP 10 (SAS Institute Inc., Cary, NC, USA).

The study was performed in accordance with European Community guidelines, and the regional ethics committees at the Karolinska Institutet and Stockholm County approved the study. Informed written consent was obtained from the parents of the enrolled patients.

RESULTS

Clinical data for patients and control groups are summarized in Table 1. There were no difference between patients and controls regarding gestational age and birth weight nor were there any difference between different HIE groups. Between the patient and the control group, there was a difference regarding the 5-min and 10-min Apgar score as well as post-partum arterial pH level (all $p < 0.05$).

Prostaglandin E_2 metabolite was compared with the Apgar score at 5 min. An Apgar score below 3 often means that immediate resuscitation is needed and is often associated with hypoxia. The overall highest measured level of PGEM (731 pg/mL) was seen in a patient with 0 Apgar at 5 min (2 at 10 min) and a fatal outcome. The Apgar score at 5 min was inversely correlated to the levels of PGEM, $r^2 = 0.208$ ($p < 0.05$) (Fig. 1A). The Apgar score at 10 min was inversely correlated to the levels of PGEM, $r^2 = 0.148$ ($p < 0.05$). Arterial pH was also negatively correlated to PGEM levels, $r^2 = 0.694$ ($p < 0.001$) (Fig. 1B).

The perinatal asphyxia patient group (n = 24) was divided into three subgroups according to Sarnat and Sarnat classification of HIE. Final outcome is summarized in Table 2. Eight infants had a mild HIE (HIE-I), and all of them had a normal outcome at follow-up. Nine infants had a moderate HIE (HIE-II). Three of these infants had an adverse neurological outcome with cerebral palsy, psychomotor retardation and seizure problems. Two infants had a mild motor impairment, and four had a normal outcome at follow-up. Seven infants had a severe HIE (HIE-III), five of these infants died on first to twelfth day of life. Two of the infants with HIE-III survived with adverse neurological outcome including spastic tetraplegia, cerebral palsy, psychomotor retardation, microcephaly and complex seizures.

Table 1 Clinical data of asphyxiated newborns and newborn nonasphyxiated controls

	Controls	HIE-I	HIE-II	HIE-III
Number of patients	11	8	9	7
Gestational age (week)*	40.6 (39.6–41.5)	40.4 (39.1–41.8)	40.4 (39.6–41.2)	40.5 (40.0–41.1)
Birthweight (g)†	3869 ± 668	3724 ± 664	3731 ± 460	3558 ± 351
Sex (female:male)	6:5	3:5	3:6	3:4
1-min Apgar score‡	8 (3–9)	2 (1–6)	1 (0–9)	2 (0–7)
5-min Apgar score‡	10 (7–10)	4 (1–7)	4 (2–10)	1 (0–9)
10-min Apgar score‡	10 (8–10)	6.5 (3–8)	6 (2–10)	3 (0–10)
Arterial pH§	7.20 (n = 6) (0.08)	7.08 (n = 6) (0.06)	6.97 (n = 9) (0.09)	6.86 (n = 6) (0.12)
Arterial base excess§	−4.4 (n = 6) (2.8)	−11 (n = 5) (3.1)	−19 (n = 8) (3.8)	−19.8 (n = 6) (2.9)
CRP§	44.9 (n = 11) (13.4)	39.6 (n = 7) (12.2)	21.8 (n = 9) (9.91)	6.5 (n = 6) (0.921)

HIE, Hypoxic–ischaemic encephalopathy.
*Mean (lower and upper 95%).
†Mean ± SD.
‡Median (range).
§Mean (SEM).

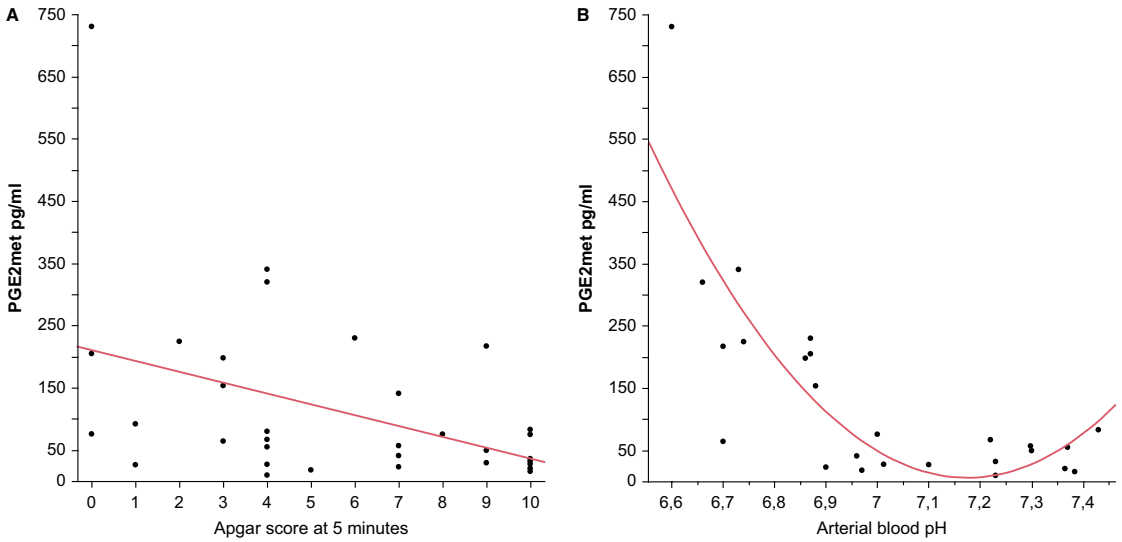

Figure 1 Prostaglandin E2 metabolite (PGEM) in cerebrospinal fluid (CSF) correlates to the 5-min Apgar and arterial pH. Relationship between PGEM levels in CSF and clinical parameters during first days of life. (A) The 5-min Apgar score was negatively related to CSF levels of PGEM ($r^2 = 0.208$, linear fit line, $p < 0.01$, $n = 34$). (B) Arterial pH at birth was indirectly correlated to PGEM levels ($r^2 = 0.694$, $p < 0.001$, $n = 27$).

Table 2 Final outcome of asphyxiated newborns and newborn nonasphyxiated controls

	Controls	HIE-I	HIE-II	HIE-III
Number of patients	11	8	9	7
Outcome				
Normal	11	8	4	0
Adverse*	0	0	5	2
Death	0	0	0	5

HIE, Hypoxic–ischaemic encephalopathy.
*Defined as neurohandicapped including microcephaly, cerebral palsy or severe EP.

The control group consisted of eleven infants where LP was performed due to initially suspected infection that was not confirmed in further analysis. All had normal outcomes.

A higher HIE score correlated to higher PGEM levels, and the HIE-III cases had significantly higher PGEM levels (242 pg/mL + 88) compared with controls (39.4 pg/mL + 6.2) and the HIE-I group (74.8 pg/mL + 27) (Fig. 2).

Three patients in the HIE-II group and one patient in the HIE-III group also had a second LP performed where sample was available for PGEM analysis. There were no large changes in levels between LP1 and LP2 except for the HIE-III case where the first LP at 6 h had 320 pg/mL PGEM and the second LP at 56 h had 70 pg/mL (Fig. 3).

A neurological assessment was carried out for all included patients and controls at three and 6 months of

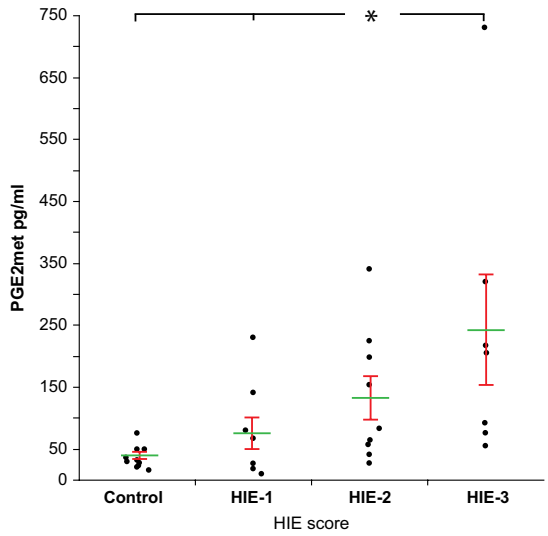

Figure 2 Prostaglandin E_2 metabolite (PGEM) in cerebrospinal fluid (CSF) correlates to the degree of hypoxic–ischaemic encephalopathy. The degree of Hypoxic–ischaemic encephalopathy (HIE) was classified as mild (HIE-I, n = 8), moderate (HIE-II, n = 9) or severe (HIE-III, n = 7). PGE_2 metabolite levels (pg/mL) in the HIE-III group were higher compared with controls and the HIE-I group. The long horizontal (green) line indicates mean value for each group, and the whiskers (red) indicate the standard error of the mean (SEM) (* = $p < 0.01$).

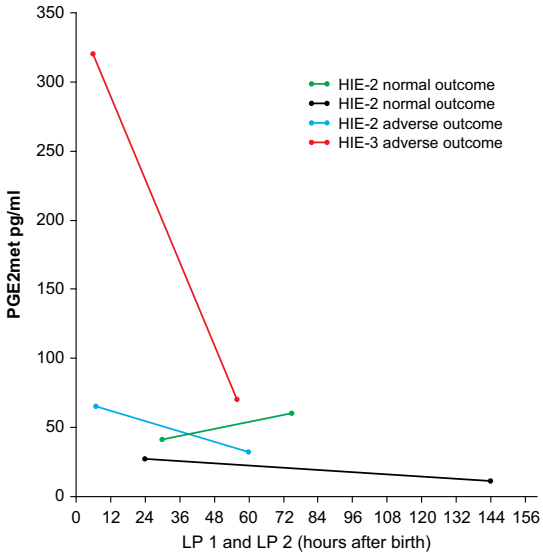

Figure 3 Kinetics of prostaglandin E_2 metabolite (PGEM) in cerebrospinal fluid (CSF). Relationship between PGEM levels in CSF during first day of life and after a secondary lumbar puncture (LP) at indicated time point. Four patients with a secondary LP had material available for analysis. Line and colour indicate samples from the same donor. Stable levels were seen in three of the samples but a rapid decline was seen in a patient with Hypoxic–ischaemic encephalopathy (HIE)-III with initial high level of PGEM.

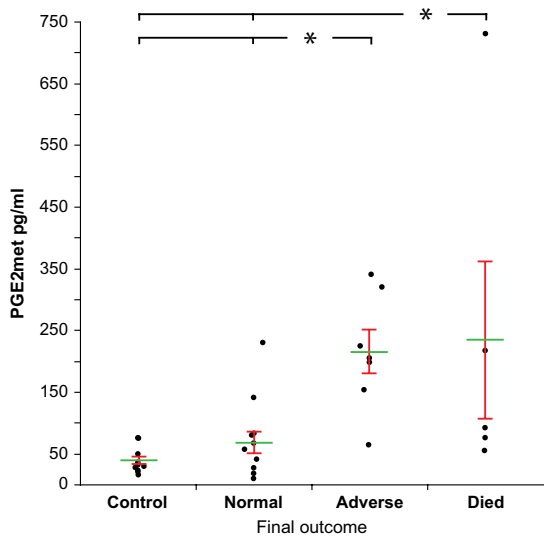

Figure 4 Prostaglandin E_2 metabolite (PGEM) in cerebrospinal fluid (CSF) correlates to the final outcome after perinatal asphyxia. Asphyxiated infants with adverse (18th-month assessment) or fatal outcome had higher levels of PGEM in CSF compared with controls and compared with asphyxiated infants with normal outcome. Values are expressed in pg/mL. The long horizontal (green) line indicates mean value for each group, and the whiskers (red) indicate the standard error of the mean (SEM) (* = $p < 0.05$).

age and a final outcome assessment at 18 months of age. Amongst the HIE patient group, the one with adverse outcome including CP, psychomotor retardation and death had significantly higher PGEM levels (223 pg/mL + 53) compared with both the patient group with normal outcome (67.2 pg/mL + 18) and control group (39 pg/mL + 6.2) (Fig. 4).

A receiver operating characteristic curve generated for PGEM and the calculated overall discriminative power using area under the curve (AUC) showed that PGEM levels have higher AUC 89.9% (Fig. 5) than both pH and BE, 87.9% and 79.7%, respectively (Figs S1–S3). At the indicated cut-off value of 80 pg/mL PGEM in CSF, the sensitivity was 85% and the specificity 83%.

DISCUSSION

The present data indicate that PGE_2 metabolite in CSF (PGEM) is a biomarker for the severity of perinatal asphyxia and long-term outcome.

We show here that PGEM correlates to the base excess and arterial pH (Fig. 1). Both are well-established and objective determinants of the foetal metabolic condition and degree of perinatal hypoxic exposure in the newborn. Thus, the data from mice where acute anoxia induces an immediate increase in mPGES-1 activity and subsequent PGE_2 release (7,22) also seem valid in human neonates. Previous investigations regarding prostaglandins and birth asphyxia have either detected no difference in CSF PGE_2 (18) or an increase in PGE_2 but not significant regarding long-term outcome or regarding the degree of HIE (23) in asphyxiated neonates compared with control newborn. This could be due to a lower number of newborn infants included, seven with moderate HIE and only three with severe HIE (23) or, more likely, that the rapid turnover and metabolism of PGE_2 into its metabolites make it hard to detect if samples are not acquired and stored rapidly. When measuring a biomarker with a short half-life, it is important to sample and store it properly, but also to recognize that it is only showing a snapshot of an ongoing process. The recorded level is one time point in a kinetic cascade. By choosing to study a more stable metabolite of PGE_2 in CSF, we detect and quantify less transient levels than of the native PGE_2 effector molecule, thereby enabling a larger window of observation. This makes the use of the more stable biomarker PGEM feasible in a clinical setting.

In addition, the PGEM levels correlate to the degree of hypoxic–ischaemic encephalopathy (Fig. 2), and importantly, PGEM correlates to the final outcome after perinatal asphyxia (Fig. 4).

From experimental studies, we know that oxygen deprivation in itself causes an immediate release of PGE_2, within minutes (5,7,22). It is likely that the high levels of PGEM found at the earliest time points in this study (Fig. 3) reflect

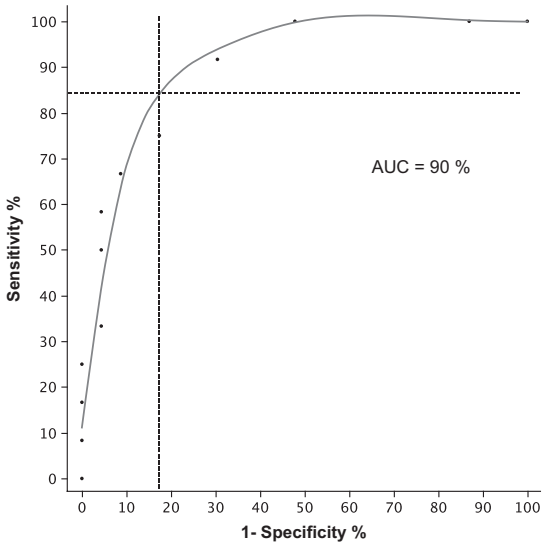

Figure 5 Receiver operating characteristic (ROC) curve of prostaglandin E_2 metabolite (PGEM) levels in relation to final outcome (adverse or normal). Adverse outcome was defined as: cerebral palsy, microcephaly and mental retardation, seizure disorder and neurodevelopment delay or death. The dashed lines show optimal discriminating cut-off value of 80 pg/mL PGEM in cerebrospinal fluid (CSF). At the indicated cut-off, the sensitivity was 85% and specificity 83%. Area under the curve (AUC) was 90% showing discriminative power.

a primary PGE_2 release following a prolonged and stressful delivery and not the secondary inflammatory response (24). This is further strengthened by measurement of PGEM after 72 h of postnatal age (Fig. 3), where PGEM decreases after birth, unless an insult such as sepsis or meningitis occurs resulting in a rapid increase in PGEM levels (data not shown). Biomarkers of birth asphyxia have been extensively studied (e.g. 25,26), but most biomarkers have been related to cellular or neuronal damage of the hypoxic–ischaemia, systemic inflammation and markers thereof (e.g. 27,28). A few studies have included PGE_2 and other eicosanoids but they have not analysed PGE2 metabolite. The rapid synthesis of PGE_2 in response to cytokine and hypoxic stimulation may make it particularly useful in the diagnosis and surveillance of infants who have been exposed to perinatal asphyxia. Does PGEM correlate better with degree of HIE and could this biomarker predict long-term outcome, thereby helping in therapeutic decisions and management of the neonate? Umbilical cord acid–base analysis and the base deficit, derived from pH and pCO2, are essential criteria for defining intrapartum hypoxia (29). An umbilical arterial pH <7 is associated with approximately 23% of neonatal neurological morbidity (1). An umbilical arterial base deficit ≥12 mmol/L and increasing levels of metabolic acidosis are associated with severity of newborn complications (29,30). Compared with these well-established acid–base balance indicators for metabolic acidosis and variables associated with long-term outcome, PGEM seems to do well (Fig. 5) (29). In this study, the ROC curve generated, and the overall discriminative power estimated by the area AUC showed that PGEM levels had a high predictive value with an AUC of 90% (Fig. 5). This was higher than both pH and BE, 88% and 80%, respectively (Fig. S1–S3). A value of less than 75% is generally regarded as not useful in clinical practice, and an optimal useful discriminative value can be over 97%. An AUC of 90% is showing a reasonable power of discrimination and indicates a potential usage, when available, as a prognostic biomarker showing risk of adverse outcome. This may be instructive in decision for postasphyxia interventions together with other clinical values. PGEM in combination with other biomarkers likely renders a higher predictive power, this need to be examined in a larger patient population. None of the neonates underwent therapeutic hypothermia treatment, as this was not an established therapy during the recruitment of this patient cohort. Therapeutic hypothermia is now established in most NICUs in OECD (Organisation for Economic Co-operation and Development) countries. Thus, the present result should also be examined in infants where therapeutic hypothermia has been used.

Further studies to evaluate the potential diagnostic benefits of monitoring PGE_2 and its metabolites compared with and together with other biomarkers are necessary. Nonetheless, the present study indicates that massive amounts of PGE_2 are released into the CSF during birth asphyxia and that the PGE_2 metabolite correlates to the degree of HIE and long-term outcome. This could make it a valuable tool in the management of neonates and decisions regarding their therapeutic support.

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References

1. Graham EM, Ruis KA, Hartman AL, Northington FJ, Fox HE. A systematic review of the role of intrapartum hypoxia-ischemia in the causation of neonatal encephalopathy. *Am J Obstet Gynecol* 2008; 199: 587–95.
2. Volpe JJ. Perinatal brain injury: from pathogenesis to neuroprotection. *Ment Retard Dev Disabil Res Rev* 2001; 7: 56–64.
3. Hanrahan JD, Cox IJ, Azzopardi D, Cowan FM, Sargentoni J, Bell JD, et al. Relation between proton magnetic resonance spectroscopy within 18 hours of birth asphyxia and neurodevelopment at 1 year of age. *Dev Med Child Neurol* 1999; 41: 76–82.
4. Thornton C, Rousset CI, Kichev A, Miyakuni Y, Vontell R, Baburamani AA, et al. Molecular mechanisms of neonatal brain injury. *Neurol Res Int* 2012; 2012: 506320.

5. Ek M, Engblom D, Saha S, Blomqvist A, Jakobsson PJ, Ericsson-Dahlstrand A. Inflammatory response: pathway across the blood-brain barrier. *Nature* 2001; 410: 430–1.
6. Herlenius E. An inflammatory pathway to apnea and autonomic dysregulation. *Respir Physiol Neurobiol* 2011; 178: 449–57.
7. Hofstetter AO, Saha S, Siljehav V, Jakobsson PJ, Herlenius E. The induced prostaglandin E2 pathway is a key regulator of the respiratory response to infection and hypoxia in neonates. *Proc Natl Acad Sci U S A* 2007; 104: 9894–9.
8. Shohami E, Gross J. Effects of hypoxia and anoxia on the ex vivo release of prostaglandins from mouse cortical slices. *J Neurochem* 1986; 47: 1678–81.
9. Allen LG, Louis TM, Kopelman AE. Brain prostaglandins E2 and F2 alpha following neonatal asphyxia in the guinea pig. *Biol Neonate* 1982; 42: 8–14.
10. Olsson A, Kayhan G, Lagercrantz H, Herlenius E. IL-1 beta depresses respiration and anoxic survival via a prostaglandin-dependent pathway in neonatal rats. *Pediatr Res* 2003; 54: 326–31.
11. Mitchell MD, Lucas A, Etches PC, Brunt JD, Turnbull AC. Plasma prostaglandin levels during early neonatal life following term and pre-term delivery. *Prostaglandins* 1978; 16: 319–26.
12. Sellers SM, Hodgson HT, Mitchell MD, Anderson AB, Turnbull AC. Raised prostaglandin levels in the third stage of labor. *Am J Obstet Gynecol* 1982; 144: 209–12.
13. Reese J, Paria BC, Brown N, Zhao X, Morrow JD, Dey SK. Coordinated regulation of fetal and maternal prostaglandins directs successful birth and postnatal adaptation in the mouse. *Proc Natl Acad Sci U S A*. 2000; 97: 9759–64.
14. Herlenius E, Lagercrantz H. Development of neurotransmitter systems during critical periods. *Exp Neurol* 2004; 190(Suppl. 1): S8–21.
15. Majed BH, Khalil RA. Molecular mechanisms regulating the vascular prostacyclin pathways and their adaptation during pregnancy and in the newborn. *Pharmacol Rev* 2012; 64: 540–82.
16. McCullough L, Wu L, Haughey N, Liang X, Hand T, Wang Q, et al. Neuroprotective function of the PGE2 EP2 receptor in cerebral ischemia. *J Neurosci* 2004; 24: 257–68.
17. Kawano T, Anrather J, Zhou P, Park L, Wang G, Frys KA, et al. Prostaglandin E2 EP1 receptors: downstream effectors of COX-2 neurotoxicity. *Nat Med* 2006; 12: 225–9.
18. Vilanova JM, Figueras-Aloy J, Rosello J, Gomez G, Gelpi E, Jimenez R. Arachidonic acid metabolites in CSF in hypoxic-ischaemic encephalopathy of newborn infants. *Acta Paediatr* 1998; 87: 588–92.
19. Granstrom E, Hamberg M, Hansson G, Kindahl H. Chemical instability of 15-keto-13,14-dihydro-PGE2: the reason for low assay reliability. *Prostaglandins* 1980; 19: 933–57.
20. Bothwell W, Verburg M, Wynalda M, Daniels EG, Fitzpatrick FA. A radioimmunoassay for the unstable pulmonary metabolites of prostaglandin E1 and E2: an indirect index of their *in vivo* disposition and pharmacokinetics. *J Pharmacol Exp Ther* 1982; 220: 229–35.
21. Sarnat HB, Sarnat MS. Neonatal encephalopathy following fetal distress. A clinical and electroencephalographic study. *Arch Neurol* 1976; 33: 696–705.
22. Siljehav V, Olsson Hofstetter A, Jakobsson PJ, Herlenius E. mPGES-1 and prostaglandin E2: vital role in inflammation, hypoxic response, and survival. *Pediatr Res* 2012; 72: 460–7.
23. Sumanovic-Glamuzina D, Culo F, Culo MI, Konjevoda P, Bozic T, Robovic A, et al. Vasodilatory prostaglandins in perinatal hypoxic brain damage. *Coll Antropol* 2008; 32(Suppl. 1): 183–7.
24. Degi R, Bari F, Thrikawala N, Beasley TC, Thore C, Louis TM, et al. Effects of anoxic stress on prostaglandin H synthase isoforms in piglet brain. *Brain Res Dev Brain Res* 1998; 107: 265–76.
25. Karlsson M, Wiberg-Itzel E, Chakkarapani E, Blennow M, Winbladh B, Thoresen M. Lactate dehydrogenase predicts hypoxic ischaemic encephalopathy in newborn infants: a preliminary study. *Acta Paediatr* 2010; 99: 1139–44.
26. Roka A, Beko G, Halasz J, Toldi G, Lakatos P, Azzopardi D, et al. Changes in serum cytokine and cortisol levels in normothermic and hypothermic term neonates after perinatal asphyxia. *Inflamm Res* 2013; 62: 81–7.
27. Blennow M, Savman K, Ilves P, Thoresen M, Rosengren L. Brain-specific proteins in the cerebrospinal fluid of severely asphyxiated newborn infants. *Acta Paediatr* 2001; 90: 1171–5.
28. Martin-Ancel A, Garcia-Alix A, Pascual-Salcedo D, Cabanas F, Valcarce M, Quero J. Interleukin-6 in the cerebrospinal fluid after perinatal asphyxia is related to early and late neurological manifestations. *Pediatrics* 1997; 100: 789–94.
29. ACOG Committee on Obstetric Practice. ACOG Committee Opinion No. 348, November 2006: Umbilical cord blood gas and acid-base analysis. *Obstet Gynecol* 2006; 108: 1319–22.
30. Low JA, Lindsay BG, Derrick EJ. Threshold of metabolic acidosis associated with newborn complications. *Am J Obstet Gynecol* 1997; 177: 1391–4.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1 Receiver-operator-characteristic (ROC) curve of pH levels in relation to final outcome (adverse or normal).
Figure S2 Receiver-operator-characteristic (ROC) curve of base excess (BE) levels in relation to final outcome (adverse or normal).
Figure S3 Receiver-operator-characteristic (ROC) curve of PGEM, pH and base excess (BE) levels in relation to final outcome (adverse or normal).

Paper II

Prostaglandin E_2 Mediates Cardiorespiratory Disturbances during Infection in Neonates

Veronica Siljehav, MD, PhD*, Annika M. Hofstetter, MD, PhD*, Kristin Leifsdottir, MD, and Eric Herlenius, MD, PhD

Objective To determine whether infection, with associated eicosanoid release, is a main cause of respiratory disruption in neonates, by measuring levels of prostaglandin E_2 (PGE_2) and its metabolite (PGEM) in cerebrospinal fluid (CSF).

Study design Of 59 eligible infants, 25 preterm infants (mean gestational age, 28 ± 0.5 weeks) and 22 full-term infants (mean gestational age, 40 ± 0.5 weeks) from a level 3 neonatal intensive care unit and the general maternity neonatal ward were enrolled prospectively. Infants with a condition that can cause secondary apnea were excluded. Cardiorespiratory disturbances, such as apnea, bradycardia, and desaturation (ABD) events, were quantified. All infants were subjected to standard laboratory analysis of blood and CSF concentrations of biomarkers, including PGE_2 and PGEM, within 24 hours of lumbar puncture, which were correlated with ABD events and culture-verified infections.

Results PGEM levels were highest in infants with culture-verified sepsis and meningitis ($P < .01$). In infants without culture-verified bacterial infections, PGEM levels were higher in preterm infants compared with term infants ($P < .05$). The numbers of desaturation events and apnea events in neonates were positively associated with PGE_2 levels in CSF ($P < .05$).

Conclusion PGE_2 and PGEM are rapidly elevated in CSF during an infectious event and may explain cardiorespiratory disturbances, which are the major presenting symptoms of neonatal infections. PGE_2 and PGEM are released during bacterial infections and could serve as biomarkers for sepsis and autonomic dysfunction in neonates. *(J Pediatr 2015; ■: ■-■).*

Sepsis and meningitis are major causes of morbidity and mortality in the neonatal population. Dominant presenting features of septicemia in preterm infants include increasing apnea (55%),[1] bradycardia, and desaturation (ABD) events.[2,3] Prostaglandin E_2 (PGE_2), induced by the proinflammatory cytokine interleukin (IL)-1β, impairs respiration during infection.[2]

IL-1β prolongs the duration of larynx stimulation-induced apnea and alters resuscitation.[4,5] The concentration of IL-1β in pharyngeal secretions from human infants is positively correlated with the clinical severity of apnea.[6] Furthermore, PGE_2 is involved in apnea in mice[7] and lambs.[8] In humans, a well-known side effect of PGE_2 therapy is apnea. Moreover, in human neonates, PGE_2 is rapidly released into the cerebrospinal fluid (CSF) during acute hypoxia and is correlated with the degree of birth asphyxia.[9]

The PGE_2 signaling pathway is induced by IL-1β binding to type I receptors on the vascular endothelial cells of the blood-brain barrier, resulting in a release of PGE_2 in the brainstem regions involved in respiratory control.[10] In turn, PGE_2 binds to the prostaglandin E receptor type 3 (EP3R) on respiratory rhythm-generating neurons in the ventrolateral medulla and depresses their activity.[11,12] An attenuated release of PGE_2 protects against respiratory depression during infectious and hypoxic events.[13]

In addition, IL-1β is elevated in some victims of sudden infant death syndrome.[5,6,14] Thus, IL-1β–induced PGE_2 release in brainstem cardiorespiratory regions mediates respiratory depression, perhaps explaining why bacterial infections are associated with this syndrome.[15]

To further explore the mediatory role of inflammation and PGE_2 in apnea and cardiorespiratory dysregulation, we investigated the association between PGE_2 and a PGE_2 metabolite (13,14-dihydro-15-keto PGE_2; herein designated PGEM) in neonatal CSF with inflammatory variables and ABD events. Our

ABD	Apnea, bradycardia, and desaturation	GMNW	General medical neonatal ward
AI	Apnea index	IL	Interleukin
CRP	C-reactive protein	NICU	Neonatal intensive care unit
CSF	Cerebrospinal fluid	PGE_2	Prostaglandin E_2
EP3R	Prostaglandin E receptor type 3	PGEM	Prostaglandin E_2 metabolite
GA	Gestational age	ROC	Receiver operating characteristic
		WBC	White blood cell

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Portions of the study were presented as a poster and abstract at the meeting of the Pediatric Academic Societies, Washington, DC, May 4-7, 2013.

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aim was to determine whether levels of PGE_2 and PGEM are associated with the severity of autonomic dysfunction during infectious events in a neonatal population.

Methods

The study was performed in accordance with European Community guidelines. The regional Ethics Committees of the Karolinska Institutet and Stockholm County approved the study. Informed written consent was obtained from parents of the enrolled patients.

A total of 59 infants from the neonatal intensive care unit (NICU) and control infants from the general medical neonatal ward (GMNW) at Karolinska University Hospital were recruited with written consent. We included 25 preterm infants (mean gestational age [GA], 28 ± 0.5 weeks; 15 males, 10 females) and 22 full-term infants (mean GA, 40 ± 0.5 weeks; 11 males, 11 females), who were enrolled prospectively (Table I). The infants underwent lumbar puncture for clinical indications, such as ABD events, suspected infection, or neurologic investigations. Infants with intraventricular hemorrhage (grade ≥2), periventricular leukomalacia, electroencephalogram-verified seizures, or major congenital abnormalities giving rise to secondary apnea were excluded. Pertinent medical information was collected, including neonatal delivery data, medical conditions, medications, laboratory data, respiratory therapy, and occurrence of ABD events. Invasive procedures, including intubation and central line placement, occurring at the time of or during the week before enrollment, were documented.

CSF Collection and Analysis

After CSF was collected for routine laboratory analysis, an additional 0.75-1.5 ml of CSF per infant was obtained for research purposes. The CSF samples were initially stored at −18°C and subsequently transferred to a −80°C freezer. Before analysis, the samples were thawed, and 20-μL aliquots were prepared.

CSF samples were analyzed for PGE_2 and PGEM using a standardized enzyme immunoassay protocol (Cayman Chemicals, Ann Arbor, Michigan).[9] Standard laboratory analysis of CSF was also performed to detect the presence of red blood cells, glucose, protein, and white blood cells (WBCs), including monocyte and leukocyte counts. Blood concentrations of infectious markers (C-reactive protein [CRP] and WBCs) were measured within 24 hours of the lumbar puncture. Serum and CSF culture results were documented.

Cardiorespiratory Recordings

Seventeen infants underwent noninvasive cardiorespiratory recording within 22 hours of lumbar puncture (mean recording time, 9:35 ± 2:27 hours) using the KIDS event monitoring system (Hoffrichter, Schwerin, Germany). CRP and Apnea Index (AI) data from some of these infants (n = 12) have been presented previously, but 3 of these infants were excluded from the present study because they did not match preset inclusion criteria.[7] Impedance pneumography detected chest wall movements as an indicator of respiratory volume changes, and a 3-lead electrocardiogram detected heart rate variability. The monitor was programmed to record all events exceeding set threshold values. An apnea-hypopnea event was defined as a ≥10-second reduction in respiratory rate by 84% from the previous mean value of 25 seconds, a preset definition in the KIDS monitoring system.[2,7] Bradycardia was defined as a heart rate of <80 bpm. Mean heart rate and respiratory frequency during the 15 and 30 seconds immediately before or apnea or bradycardia, respectively, were recorded with the KIDS monitor. The 60-second periods both before and after the events were stored in the monitor's memory.

Data and Statistical Analyses

Each cardiorespiratory recording was analyzed to assess ABD events. Events over time were defined as the AI (AI = apneas-hypopneas/hour) and the bradycardia index (bradycardias/hour).[2] Baseline respiratory frequency and heart rate were also determined. All movement artifacts were excluded from analysis. Cardiorespiratory data were correlated with infant medical data, including GA, postnatal age, presence of culture-verified bacterial infection or viral infection, medical diagnoses, and factors that could affect cytokine concentrations (ie, intubation, indomethacin therapy, perinatal asphyxia, hypoxic-ischemic encephalopathy, and corticosteroid administration). An investigator blinded to the laboratory and the eicosanoid analyses performed retrospective collection of ABD events in the abstracted medical records. Cardiorespiratory data were also correlated with CSF concentrations of PGE_2 and PGEM.

Unless stated otherwise, clinical data are presented as mean and 95% CI or IQR. Parametric clinical data were assessed

Table I. Characteristics of preterm and term infants in the NICU and term infants in the GMNW

	n	GA, wk + d	Apgar 1 score	Apgar 5 score	Apgar 10 score	Lumbar puncture, PNd	CRP, mg/L
Preterm, NICU	25	28 ± 0.5	5 ± 0.5	7 ± 0.4	8 ± 0.3	15 ± 2*	52 ± 9
Term, NICU	14	40 ± 0.6	7 ± 0.7	9 ± 0.6	9 ± 0.5	5 ± 3	83 ± 15*
Term, GMNW	8	40 ± 0.8	8 ± 0.9	9 ± 0.8	10 ± 0.6	1 ± 4	16 ± 15

PNd, postnatal day.
Apgar score tended to be lower in preterm infants in all observation periods (1, 5, and 10 minutes). Lumbar puncture was performed at a later PNd in preterm infants compared with term infants. The CRP differed between groups. Data are presented as mean ± SEM.
*$P < .05$.

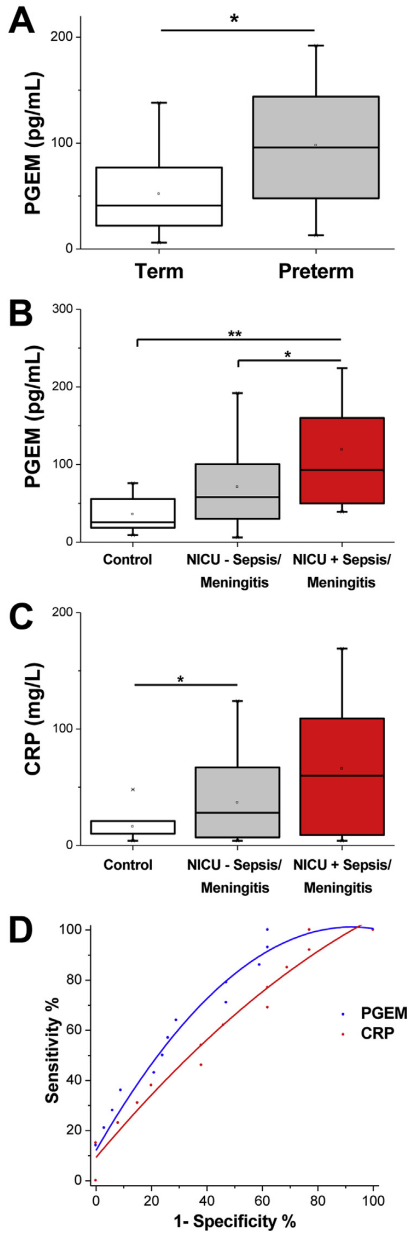

Figure 1. **A,** Comparing term and preterm infants without culture-verified bacterial infections, PGEM levels were higher in the preterm infants. PGEM levels did not differ in infants who were intubated or had a central line. **B,** PGEM levels were higher in the CSF of infants with culture-positive infections. **C,** CRP levels were highest in infants in the NICU, but could not differentiate sepsis and meningitis from other clinical cases. **D,** ROC curves show better sensitivity and specificity to infection for PGEM level compared with CRP level. Data are presented as mean ± SEM. $^{*}P < .05$; $^{**}P < .01$.

using the Student *t* test. Unequal variance was tested using Levene test, and if positive parametric data was tested by using Welch's test and nonparametric by using Wilcoxon χ^2 test. A generalized linear model was used to identify the variables associated with PGE_2 level. Correlation analyses were performed without making distinction between term and preterm infants. Receiver operating characteristic (ROC) curves were calculated to illustrate and evaluate the diagnostic (prognostic) performance of PGE_2, PGEM, and CRP in relation to ABD events and culture-verified bacterial infections. In all cases, *P* values of <.05 were considered statistically significant.

Results

Age, birth weight, and time to lumbar puncture differed between the groups. Apgar scoring was performed to assess the infants' condition at 1, 5, and 10 minutes after birth. Characteristics of the study groups are summarized in **Table I**.

Preterm infants had higher levels of PGEM relative to term infants ($P < .05$, Student *t* test) (**Figure 1**, A). PGE_2 and CRP levels were not affected by GA (**Table II**; available at www.jpeds.com), and PGEM levels were not affected by recent intubation or central catheter insertion.

Nonetheless, because of the small sample size, the results were not adjusted for GA. The highest PGE_2 and PGEM levels were seen in the infants with culture-confirmed bacterial infections. PGEM levels were higher in infants with culture-verified meningitis and sepsis compared with those without either condition in the NICU or GMNW ($P < .05$ and <.01, respectively, χ^2 test, power 75%) (**Figure 1**, B). CRP levels did not differentiate culture-confirmed bacterial infection from other infections. CRP levels were higher in infants in the NICU compared with those in the GMNW ($P < .05$, χ^2 test) (**Figure 1**, C and **Table I**). The ROC curves of PGEM and CRP levels show that PGEM level was both more specific and more sensitive in discriminating culture-positive bacterial infections from other infections (**Figure 1**, D). CSF protein levels were elevated in preterm infants compared with term infants; this difference remained after excluding infants with sepsis or meningitis owing to the potential for confounding[16] (**Tables I** and **III** and **Figure 1**, C and D; **Table III** available at www.jpeds.com). Estimation of the overall predictive value for culture-verified infection by the area under the ROC curve showed positive predictive values of 75% for PGEM level, but only 63% for CRP level. Bacteria and viral culture data are presented in **Table IV** (available at www.jpeds.com).

Both PGE_2 and PGEM levels correlated with CRP levels ($R^2 = 0.24$, linear fit line; $P < .05$; n = 28 and $R^2 = 0.24$, linear fit line; $P < .01$; n = 35, respectively) (**Figure 2**; available at www.jpeds.com). No correlations were found between serum or WBCs in the CSF and PGE_2 or PGEM levels.

Desaturation events in infants in the NICU were correlated with PGE_2 levels and tended to correlate with PGEM levels ($R^2 = 0.21$, linear fit line; $P < .01$; n = 35 and $R^2 = 0.08$, linear

fit line; $P = .08$; n = 37, respectively) (Figure 3, A and B; available at www.jpeds.com). AI, that was monitored in 8 infants, correlated with PGE2 levels ($P < .05$, $\chi 2$ test) (Figure 3, C). In addition, shorter apnea periods (10-14 seconds) tended to correlate with PGEM levels, but did not reach significance in our study population ($R^2 = 0.38$, linear fit line; $P = .06$; n = 9). The 4 infants treated with indomethacin within 6 hours before lumbar puncture had lower PGE_2 levels, but not lower PGEM levels.

Infants who experienced an ABD event before lumbar puncture had higher PGEM levels relative to infants investigated because of other indications, such as neurologic investigations or increased CRP levels ($P < .05$, Student t test) (Figure 4, A). The ROC curves of PGEM and CRP levels showed that PGEM levels were both more specific and more sensitive when correlated with ABD events (Figure 4, B). In terms of overall predictive value for ABD events, estimation by the area under the ROC curve showed positive predictive values of 78% for PGEM level, but only 59% for CRP level.

Discussion

Here we have demonstrated that in neonates, levels of PGE_2 and PGEM in CSF are inversely correlated with GA and are positively correlated with the presence and severity of ABD events and infection. These findings provide an understanding of the prostaglandin mechanism underlying autonomic dysfunction in neonates. Moreover, our data suggest that PGE_2 and PGEM may be promising novel biomarkers for infections and cardiorespiratory dysfunction in young infants.

Neonatal bacterial infections carry high rates of mortality and morbidity.[17] Early diagnosis is difficult because the initial clinical signs are nonspecific.[1] Consequently, physicians frequently prescribe antibiotic treatment for newborn infants, out of concern for possibly overlooking a life-threatening infection.[18] The initiation of treatment is often based on a combination of risk factors for infection, clinical presentation, and inflammatory markers. The duration of antibiotic therapy potentially could be minimized by adding PGEM to the decision making algorithm, particularly for infants at low risk of sepsis, as has been shown with other proinflammatory markers.[16] Blood culture is the traditional gold standard for diagnosing infections; however, obtaining results can take up to several days, and data may be inconclusive.[19] Thus, inflammatory markers are often used in making decisions regarding therapeutic interventions. Standard methods for the evaluation of inflammatory status (eg, measurement of CRP and WBC levels) have proven accurate for establishing the degree of infection, if repetitive measurements are performed days apart[20]; however, they have only limited sensitivity during the early phase of the disease.[21] Our present data also illustrate that CRP is an unreliable biomarker and cannot distinguish bacterial infection from other infections (Figure 1, C).

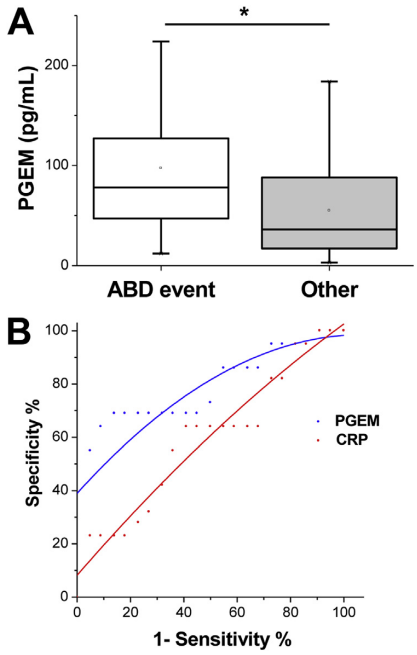

Figure 4. **A,** Infants who presented with ABD events before lumbar puncture had increased PGEM levels compared with infants investigated for other reasons. **B,** ROC curves illustrate greater sensitivity and specificity to ABD events for PGEM level compared with CRP level. Data are presented as mean ± SEM. $^*P < .05$.

Both PGEM and PGE_2 levels were associated with CRP level (Figure 2). Our results demonstrate that compared with CRP, PGEM correlates better with culture-verified infection and is better able to predict whether or not the infection is bacterial (Figure 1, B and D). However, a high PGEM level does not always indicate infection. Because of the rapid metabolism of PGE_2 in blood (ie, only 3% remains at 1.5 minutes after administration),[22] the use of blood samples is limited. PGEM has the advantage of being accumulated more stably into the bloodstream than PGE_2, but it still has a short half-life in blood compared with plasma ($t_{1/2}$ = 8 min in blood, 45 min in plasma),[22] making CSF more suitable for analysis. PGEM levels are higher than PGE_2 levels in biological fluids.[23] PGEM is catabolized enzymatically from PGE_2 by stepwise oxidation and reduction into 13,14-dihydro-15-keto PGE_2. In contrast, PGE_2 is a bioactive mediator of inflammation and has a rapid onset of action (within hours).[24] It also has a short half-life in most biological fluids ($t_{1/2}$ = 5 minutes in plasma).[25] Thus, both PGE_2 and PGEM may be useful biomarkers, more precise and more sensitive than CRP and WBCs for detecting bacterial infections. Because these molecules are detectable early in the course of illness, they can more quickly predict inflammation. A recent review

that did not evaluate PGE_2 and PGEM concluded that although some promising biomarkers may aid the diagnosis of infection, the biomarkers investigated to date are far too expensive and not sufficiently selective for discerning use.[26] We recognize that PGEM level alone is not sufficient to determine whether a neonatal infant has an infection or is prone to experience ABD events; nonetheless, our data suggest that analysis of PGEM level, in combination with analysis of established biomarkers or ABD monitoring, could be a promising biomarker to assist in the diagnosis of infection.

Apnea of prematurity, even without associated infection, is considered a risk factor for brain injury and developmental disorders in infants.[27] Neonatal sepsis is associated with decreased normal heart rate variability, even before clinical manifestations of sepsis become evident.[28] We previously described the mechanism behind inflammatory-induced apnea in neonatal mice via the PGE_2-induced pathway[7]; however, this mechanism had not been thoroughly explored in human infants until the present study. CRP level, for its part, correlates with autonomic dysfunction,[29] but the detection of CRP is delayed[30] and would not affect the decision of initial observation and treatment.

PGE_2 has evolved as a potential biomarker for autonomic dysfunction in neonates.[7] Our results suggest that PGE_2 level correlates with desaturation events, as does PGEM level, although not reaching the level of significance in the present study ($P = .08$) (**Figure 3**, A and B). This correlation is not seen with either plasma CRP or WBC count. CSF proteins and WBCs are not associated with ABD events. A limitation concerning the associations between ABD events in the infants treated in the NICU, who did not undergo a KIDS event recording, is that these data are dependent on nurse or physician surveillance and recordings documented in the patient records. Given that approximately one-half of all events are not documented, the zero values are likely the most unreliable.[31] Nonetheless, when using our more standardized KIDS event monitoring, the results support the hypothesis that PGE_2 level is directly related to and may explain the increased incidence of ABD events in the neonate during infection and other inflammatory events (**Figure 3**, C). Moreover, infants who underwent lumbar puncture because of ABD events had increased PGEM levels (**Figure 4**, A).

In the present study, we controlled for factors that could increase inflammatory variables prior to enrolment, such as intubation.[32] However, these factors did not affect the results. These findings further strengthen our hypothesis supporting the PGE_2 pathway as a key player in regulating respiration in the neonate.[7,11,13] PGE_2 impairs respiratory effort and induces apneas in neonates through its actions on the EP3R present in the brainstem respiratory-related regions.[11] Within the blood-brain barrier, PGE_2 decreases brainstem network activity through its postsynaptic actions to reduce excitatory synaptic transmission,[11] likely through PGE_2-induced hyperpolarization via EP3R expressed in neurons. PGE_2 release in the vicinity of respiratory-related neurons in the brainstem is the result of either an inflammatory response to IL-1β or hypoxia.[24,33] The expression of microsomal prostaglandin E synthase-1, the enzyme enabling PGE_2 release in the brainstem, increases during an anoxic event and can affect outcomes[13]; thus, not only PGE_2 and PGEM levels, but also microsomal prostaglandin E synthase-1 activity, might be a potential target of apnea management.[13] PGE_2 production is inhibited by indomethacin, a drug used in the NICU setting to treat patent ductus arteriosus.[34] In the present study, indomethacin lowered PGE_2 levels, as expected. PGEM levels were not affected, likely reflecting PGE_2 levels before indomethacin treatment, owing to the longer half-life of PGEM.

Preterm infants had higher PGEM levels than term infants (**Figure 1**, A). PGE_2 exerts multiple effects on the neonatal kidney, particularly on hemodynamics and the water/electrolyte balance, but also on its development.[35] Preterm infants have higher levels of urinary PGE_2,[36] and the levels also increase to a greater extent in preterm infants compared with term infants during the first days of life.[37] The increased levels of PGEM in our preterm infants also could reflect these infants' immature respiratory regulation, leading to apnea of prematurity.[38] This is because prostaglandins released by preterm-specific inflammation give rise to apneas. In addition, apneas give rise to intermittent hypoxia, and thus this condition on its own could lead to prostaglandin release.[7,13]

The present study confirms the relationships among PGE_2, PGEM, infection, and autonomic dysfunction, but further studies are needed to evaluate the potential and clinical feasibility of these two molecules as biomarkers in the diagnostic palette for infection. This should be investigated with a larger study population based on this exploratory study. Thus, the present data are encouraging but preliminary. Nonetheless, the rapid synthesis of PGE_2 in response to inflammatory cytokine stimulation makes it particularly intriguing and possibly useful in the diagnosis and surveillance of infants with infection. In addition to an immediate release, PGE_2 has a short half-life, as noted above. This property makes immediate storage of samples and prompt measurement of PGE_2 level paramount, to give an indication of present status. Despite the important role of PGE_2 during inflammation, it is not routinely used as a diagnostic tool in clinical practice because of its rapid metabolism in vivo and the inherent difficulties in measurement.

Another limitation of the present study is that PGE_2 may have been metabolized in samples that were not frozen or analyzed immediately after collection, potentially leading to underestimation of PGE_2 levels. PGEM is more stable in CSF ($t_{1/2}$ in plasma = 45 minutes) and also less sensitive to sample handling; as such, it may better reflect a recent and ongoing inflammatory process within the CSF environment. The stable metabolite of PGE_2, tetranor-PGEM, is secreted into urine and has been suggested to be a reliable inflammatory biomarker.[39] Our recent data also suggest that measurement of urinary tetranor-PGEM, which is less invasive than lumbar puncture, also may be used as a

biomarker for inflammation in infants.[40] Studies evaluating the feasibility of using urinary tetranor-PGEM in addition to 13,14-dihydro-15-keto PGE_2 as a biomarker for disease severity and kinetics in newborns and infants are currently underway.

In summary, we have demonstrated that PGE_2 and its metabolite reflect ongoing inflammation in neonates, and have suggested that the increase in PGE_2 levels can explain the cardiorespiratory disturbances that are the presenting symptoms of infection in neonatal infants. This finding opens up new avenues for early and precise diagnostic and therapeutic interventions in preterm and term infants with suspected infection. ■

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References

1. Fanaroff AA, Korones SB, Wright LL, Verter J, Poland RL, Bauer CR, et al. Incidence, presenting features, risk factors and significance of late-onset septicemia in very low birth weight infants. National Institute of Child Health and Human Development Neonatal Research Network. Pediatr Infect Dis J 1998;17:593-8.
2. Hofstetter AO, Legnevall L, Herlenius E, Katz-Salamon M. Cardiorespiratory development in extremely preterm infants: vulnerability to infection and persistence of events beyond term-equivalent age. Acta Paediatr 2008;97:285-92.
3. Sullivan BA, Grice SM, Lake DE, Moorman JR, Fairchild KD. Infection and other clinical correlates of abnormal heart rate characteristics in preterm infants. J Pediatr 2014;164:775-80.
4. Froen JF, Akre H, Stray-Pedersen B, Saugstad OD. Adverse effects of nicotine and interleukin-1beta on autoresuscitation after apnea in piglets: implications for sudden infant death syndrome. Pediatrics 2000;105:e52.
5. Stoltenberg L, Sundar T, Almaas R, Storm H, Rognum TO, Saugstad OD. Changes in apnea and autoresuscitation in piglets after intravenous and intrathecal interleukin-1 beta injection. J Perinat Med 1994;22:421-32.
6. Lindgren C, Grögaard J. Reflex apnoea response and inflammatory mediators in infants with respiratory tract infection. Acta Paediatr 1996;85:798-803.
7. Hofstetter AO, Saha S, Siljehav V, Jakobsson PJ, Herlenius E. The induced prostaglandin E2 pathway is a key regulator of the respiratory response to infection and hypoxia in neonates. Proc Natl Acad Sci U S A 2007;104:9894-9.
8. Alvaro RE, Hasan SU, Chemtob S, Qurashi M, Al-Saif S, Rigatto H. Prostaglandins are responsible for the inhibition of breathing observed with a placental extract in fetal sheep. Respir Physiol Neurobiol 2004;144:35-44.
9. Björk L, Leifsdottir K, Saha S, Herlenius E. PGE-metabolite levels in CSF correlate to HIE score and outcome after perinatal asphyxia. Acta Paediatr 2013;102:1041-7.
10. Van Dam AM, De Vries HE, Kuiper J, Zijlstra FJ, De Boer AG, Tilders FJ, et al. Interleukin-1 receptors on rat brain endothelial cells: a role in neuroimmune interaction? FASEB J 1996;10:351-6.
11. Siljehav V, Shvarev Y, Herlenius E. Il-1ß and prostaglandin E2 attenuate the hypercapnic as well as the hypoxic respiratory response via prostaglandin E receptor type 3 in neonatal mice. J Appl Physiol 2014;117:1027-36.
12. Ek M, Arias C, Sawchenko P, Ericsson-Dahlstrand A. Distribution of the EP3 prostaglandin E(2) receptor subtype in the rat brain: relationship to sites of interleukin-1–induced cellular responsiveness. J Comp Neurol 2000;428:5-20.
13. Siljehav V, Olsson Hofstetter A, Jakobsson PJ, Herlenius E. mPGES-1 and prostaglandin E2: vital role in inflammation, hypoxic response, and survival. Pediatr Res 2012;72:460-7.
14. Guntheroth WG. Interleukin-1 as intermediary causing prolonged sleep apnea and SIDS during respiratory infections. Med Hypotheses 1989;28:121-3.
15. Weber MA, Klein NJ, Hartley JC, Lock PE, Malone M, Sebire NJ. Infection and sudden unexpected death in infancy: a systematic retrospective case review. Lancet 2008;371:1848-53.
16. Polin RA , Committee on Fetus and Newborn. Management of neonates with suspected or proven early-onset bacterial sepsis. Pediatrics 2012;129:1006-15.
17. Weston EJ, Pondo T, Lewis MM, Martell-Cleary P, Morin C, Jewell B, et al. The burden of invasive early-onset neonatal sepsis in the United States, 2005-2008. Pediatr Infect Dis J 2011;30:937-41.
18. Franz AR, Bauer K, Schalk A, Garland SM, Bowman ED, Rex K, et al. Measurement of interleukin 8 in combination with C-reactive protein reduced unnecessary antibiotic therapy in newborn infants: a multicenter, randomized, controlled trial. Pediatrics 2004;114:1-8.
19. Connell TG, Rele M, Cowley D, Buttery JP, Curtis N. How reliable is a negative blood culture result? Volume of blood submitted for culture in routine practice in a children's hospital. Pediatrics 2007;119:891-6.
20. Benitz WE, Han MY, Madan A, Ramachandra P. Serial serum C-reactive protein levels in the diagnosis of neonatal infection. Pediatrics 1998;102:e41.
21. Mussap M. Laboratory medicine in neonatal sepsis and inflammation. J Matern Fetal Neonatal Med 2012;25(Suppl 4):32-4.
22. Hamberg M, Samuelsson B. On the metabolism of prostaglandins E1 and E2 in man. J Biol Chem 1971;246:6713-21.
23. Bothwell W, Verburg M, Wynalda M, Daniels EG, Fitzpatrick FA. A radioimmunoassay for the unstable pulmonary metabolites of prostaglandin E1 and E2: an indirect index of their in vivo disposition and pharmacokinetics. J Pharmacol Exp Ther 1982;220:229-35.
24. Ek M, Engblom D, Saha S, Blomqvist A, Jakobsson PJ, Ericsson-Dahlstrand A. Inflammatory response: pathway across the blood-brain barrier. Nature 2001;410:430-1.
25. Granström E, Hamberg M, Hansson G, Kindahl H. Chemical instability of 15-keto-13,14-dihydro-PGE2: the reason for low assay reliability. Prostaglandins 1980;19:933-57.
26. Srinivasan L, Harris MC. New technologies for the rapid diagnosis of neonatal sepsis. Curr Opin Pediatr 2012;24:165-71.
27. Janvier A, Khairy M, Kokkotis A, Cormier C, Messmer D, Barrington KJ. Apnea is associated with neurodevelopmental impairment in very low birth weight infants. J Perinatol 2004;24:763-8.
28. Griffin MP, O'Shea TM, Bissonette EA, Harrell FE Jr, Lake DE, Moorman JR. Abnormal heart rate characteristics preceding neonatal sepsis and sepsis-like illness. Pediatr Res 2003;53:920-6.
29. Tauman R, Ivanenko A, O'Brien LM, Gozal D. Plasma C-reactive protein levels among children with sleep-disordered breathing. Pediatrics 2004;113:e564-9.
30. Pourcyrous M, Korones SB, Arheart KL, Bada HS. Primary immunization of premature infants with gestational age <35 weeks: cardiorespiratory complications and C-reactive protein responses associated with administration of single and multiple separate vaccines simultaneously. J Pediatr 2007;151:167-72.

31. Vergales BD, Paget-Brown AO, Lee H, Guin LE, Smoot TJ, Rusin CG, et al. Accurate automated apnea analysis in preterm infants. Am J Perinatol 2014;31:157-62.
32. Puyo CA, Tricomi SM, Dahms TE. Early biochemical markers of inflammation in a swine model of endotracheal intubation. Anesthesiology 2008;109:88-94.
33. Li W, Wu S, Hickey RW, Rose ME, Chen J, Graham SH. Neuronal cyclooxygenase-2 activity and prostaglandins PGE2, PGD2, and PGF2 alpha exacerbate hypoxic neuronal injury in neuron-enriched primary culture. Neurochem Res 2008;33:490-9.
34. Pacifici GM. Clinical pharmacology of ibuprofen and indomethacin in preterm infants with patent ductus arteriosus. Curr Pediatr Rev 2014; 10:216-37.
35. Antonucci R, Cuzzolin L, Arceri A, Fanos V. Urinary prostaglandin E2 in the newborn and infant. Prostaglandins Other Lipid Mediat 2007;84: 1-13.
36. Joppich R, Kiemann U, Mayer G, Häberle D. Effect of antidiuretic hormone upon urinary concentrating ability and medullary c-AMP formation in neonatal piglets. Pediatr Res 1979;13:884-8.
37. Agostiniani R, Mariotti P, Cataldi L, Fanos V, Sani S, Zaccaron A, et al. Role of renal PGE2 in the adaptation from foetal to extrauterine life in term and preterm infants. Prostaglandins Leukot Essent Fatty Acids 2002;67:373-7.
38. Di Fiore JM, Martin RJ, Gauda EB. Apnea of prematurity: perfect storm. Respir Physiol Neurobiol 2013;189:213-22.
39. Arai Y, Arihiro S, Matsuura T, Kato T, Matsuoka M, Saruta M, et al. Prostaglandin E-major urinary metabolite as a reliable surrogate marker for mucosal inflammation in ulcerative colitis. Inflamm Bowel Dis 2014;20:1208-16.
40. Idborg H, Pawelzik SC, Perez-Manso M, Björk L, Hamrin J, Herlenius E, et al. Evaluation of urinary prostaglandin E2 metabolite as a biomarker in infants with fever due to viral infection. Prostaglandins Leukot Essent Fatty Acids 2014;91:269-75.

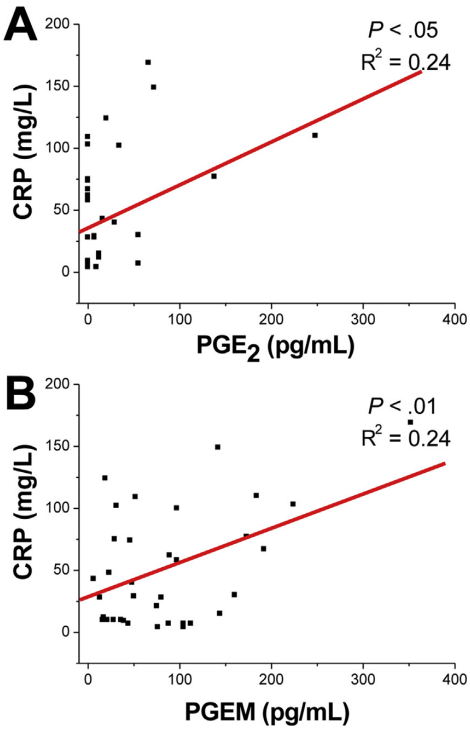

Figure 2. Relationships between PGE_2 and PGEM levels and CRP levels in the CSF. **A,** CRP levels were measured just before lumbar puncture (mean, 5.6 ± 0.7 hours) and showed a positive association with PGE_2 levels. **B,** CRP levels also correlated with PGEM levels both using linear fit (*red line*) and 2 degree polynominal fit (not shown).

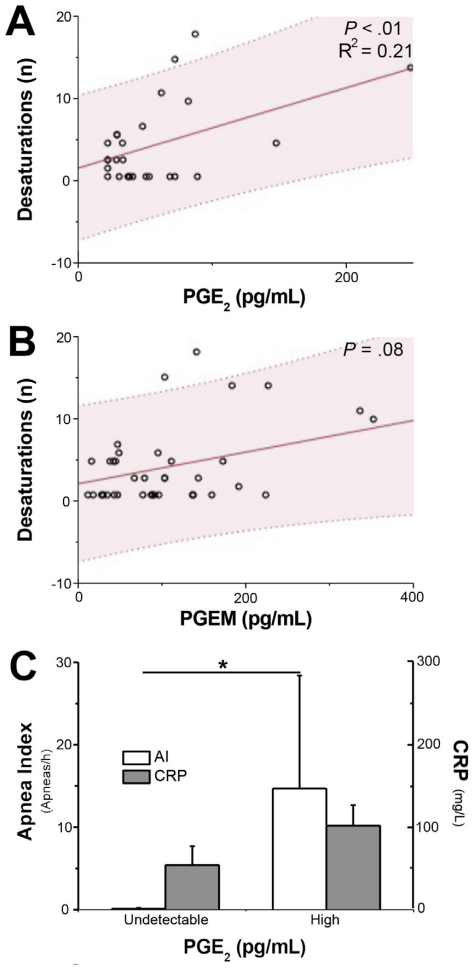

Figure 3. **A,** Number of desaturations in infants in the NICU correlated with PGE_2 levels. **B,** PGEM levels and desaturations also tended to be correlated, but the results did not reach significance. **C,** Apnea in infants recorded by the KIDS event monitoring system showed a correlation between AI and PGE_2 levels. Data are presented as mean ± SEM. *$P < .05$.

Table II. Characteristics of preterm and term infants, excluding all infants with sepsis or meningitis

	n	GA, wk + d	Apgar 1 score	Apgar 5 score	Apgar 10 score	Lumbar puncture, PNd	CRP, mg/L
Preterm	10	27 ± 0.5*	5 ± 0.9	6 ± 0.6	8 ± 0.6	16 ± 2*	34 ± 9
Term	20	40 ± 0.4	7 ± 0.6	9 ± 0.5	9 ± 0.4	2 ± 1	49 ± 12

PNd, postnatal day.
Preterm vs term infants showed a tendency toward lower Apgar scores throughout the first 10 minutes of life, with the lowest scores seen at 1 minute (Apgar 1) and somewhat higher scores seen at 10 minutes (Apgar 10). Lumbar puncture was performed on a later PNd in preterm infants. Data are presented as mean ± SEM.
*$P < .05$.

Table III. Characteristics of inflammatory variables in blood and CSF of preterm and term infants

	n	WBC, serum, 10^9/L	WBC, CSF, 10^6/L	Monocytes, CSF, 10^6/L	Protein, CSF, g/L
Preterm	19	13 ± 2.2	13 ± 5.5	11 ± 8.0	1.7 ± 0.1*
Term	22	13 ± 2.5	26 ± 14	15 ± 6.7	0.9 ± 0.1

No difference in WBCs in either serum or CSF was seen between preterm and term infants; however, protein levels in CSF were higher in preterm infants. Data are presented as mean ± SEM.
*$P < .05$.

Table IV. Characteristics of bacterial and viral agents in blood and CSF cultures

	n	Positive/negative	Pathogen
Bacteria	48	Positive: 13 Negative: 35	CoNS: 6 *Staphylococcus aureus*: 2 *Escherichia coli*: 2 Enterococci: 1 Gram-negative streptococci: 1 Group B streptococci: 1
Virus	22	Positive: 1 Negative: 21	Cytomegalovirus: 1

CoNS, = Coagulase-negative staphylococci.
All of the infants were cultured for bacteria blood, CSF, or both. A significant amount of bacteria was found in 13 of 48 infants, with CoNS the most prevalent bacteria. Virus was cultured from CSF and blood in 22 of 26 infants. Five infants underwent viral examination of the nasopharynx, which revealed no virus.

Paper III

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Fas-ligand and interleukin-6 in the cerebrospinal fluid are early predictors of hypoxic-ischemic encephalopathy and long-term outcomes after birth asphyxia in term infants

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Abstract

Background: Cerebral ischemia generates neuroinflammation that can induce neural cell death. This cohort study assessed whether Fas-ligand (FasL) and interleukin (IL)-6 levels in the cerebrospinal fluid (CSF) after hypoxic-ischemic encephalopathy (HIE) can serve as biomarkers of hypoxic brain injury in neonates.

Methods: Term infants (> 37-week gestational age) who were admitted to the neonatal intensive care unit of Karolinska University Hospital in years 2002 to 2004 with perinatal asphyxia were enrolled prospectively. Control infants without brain pathology underwent lumbar puncture for suspected infection. FasL and IL-6 levels were measured in the CSF, by enzyme-linked immunosorbent assays. All patients underwent neurological assessment at 18 months. HIE was classified as mild, moderate, or severe (HIE I–III). Adverse neurological outcome at 18 months was defined as a mental developmental index < 85, deafness, blindness, cerebral palsy, or seizure disorder.

Results: Of the 44 HIE patients, 14, 16, and 14 had HIE-I, HIE-II, and HIE-III, respectively. HIE-II and HIE-III patients had higher FasL and IL-6 levels than HIE-I patients and the 20 controls (all $p < 0.0001$). Patients with adverse outcomes had higher FasL and IL-6 levels than patients with normal outcomes and controls (both $p < 0.0001$). On receiver-operator curve analyses, FasL and IL-6 (alone and together) were highly predictive of HIE grade and outcome (areas under the curve range 0.86–0.94) and showed high sensitivity (66.7–100%). These biomarkers performed better than cord blood pH (areas under the curve: HIE grade = 0.80, adverse outcomes = 0.86).

Conclusion: CSF biomarkers FasL and IL-6 predicted severity of encephalopathy and long-term outcomes in post-asphyxiated infants better than a standard biomarker.

Keywords: Asphyxia, Biomarker, Hypoxic-ischemic encephalopathy, Interleukin-6, Fas-ligand, Predictive power

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Key notes

- Following birth asphyxia, FasL and IL-6 are released into the CSF.
- FasL and IL-6 levels in CSF correlate with HIE grade and long-term clinical outcome of post-asphyxiated patients.
- Both alone and together, FasL and IL-6, accurately predict the degree of hypoxic neonatal brain injury and long-term outcomes with high sensitivity.

Background

Hypoxic-ischemic encephalopathy (HIE) is characterized by clinical and laboratory signs of brain damage after perinatal asphyxia. It has an incidence of 2.5 per 1000 live births and associates with high rates of morbidity and mortality [1]. Cerebral hypoxic ischemia induces a strong neuroinflammatory response. After the primary insult, a cascade of events evolves to delayed cellular death that extends over several days. Apoptosis features particularly prominently in this phenomenon [2]. This biphasic pattern of neuronal death represents a window of opportunity for treatment such as therapeutic hypothermia, which is today the standard treatment for neonates with HIE [3]. However, while hypothermia is an effective treatment strategy, there is still an urgent need for additional novel interventions that protect the neurons from secondary damage and improve the clinical outcome.

The pathophysiology of HIE is not fully understood, but several studies suggest that the extrinsic apoptotic pathway involving the transmembrane death receptor Fas (CD95/Apo-1) and its natural ligand Fas-ligand (FasL) plays a central role [2, 4]. This is supported by several other studies. First, Fas receptor expression is upregulated in the brain during hypoxia, and mice that lack functional Fas receptors are protected from HIE brain injury [5]. Second, after head trauma in adults, Fas receptor and FasL are expressed in the central nervous system [6]. Third, preterm infants with post-hemorrhagic hydrocephalus have increased levels of soluble Fas receptor (sFas) in the cerebrospinal fluid (CSF), and the concentration correlates with the extent of white matter damage [7].

Evidence from experimental research indicates that inflammation and the associated production of inflammatory mediators play a significant role in the pathophysiology of brain ischemia [8]. Interleukin-6 (IL-6) is particularly interesting because its production in the post-hypoxic inflammatory cascade has been reported to have both neurotoxic and neuroprotective effects [9, 10]. The neurotoxicity of IL-6 is demonstrated by the fact that, in infants with HIE, increased IL-6 levels, in both serum and CSF, correlate positively with brain injury severity and the clinical outcome [11, 12]. The neuroprotective effect of IL-6 is exemplified by its ability to protect cerebral granular neurons from *N*-methyl-D-aspartate-induced excitotoxicity in vitro (9) and the fact that IL-6 injections into the brain after ischemia reduce ischemic brain injury (10). These opposing roles of IL-6 may reflect different functions at different stages of brain ischemia damage: specifically, it may mediate destructive inflammation during the acute phase while enhancing regeneration of the nerves in the subacute and prolonged phases [10, 13].

We hypothesized that FasL and IL-6 are upregulated in CSF samples from human infants after perinatal asphyxia and that they may correlate positively with the severity of HIE and the clinical outcome of patients. To address this, infants with HIE were compared with control infants with suspected infection in terms of FasL and IL-6 levels in the CSF.

Methods

Ethics

This study was performed in accordance with the tenets of the declaration of Helsinki 1975 and its revision in 1983 and European Community guidelines. The regional ethics committees at the Karolinska Institutet and the Stockholm County approved the study (Dnr 98-246, 2003-174, 2011/1891-31). Informed written consent was obtained from the parents of the enrolled patients.

Patient population

All consecutive term infants (> 37-week gestational age) who were admitted to the neonatal intensive care unit of Karolinska University Hospital in Stockholm between October 2000 and September 2004 were enrolled prospectively into the study if they underwent clinically indicated lumbar puncture (LP), had experienced perinatal asphyxia, and met the following criteria:

1. Signs of fetal distress, as indicated by the cardiotocographic pattern of late decelerations, lack of variability, or bradycardia; meconium staining of amniotic fluid; and scalp pH < 7.1 or blood lactate levels > 4.8 mM.
2. Postnatal stress, as indicated by Apgar score < 6 at 5 min or pH ≤ 7.00/base deficit ≥ 16 mEq in the umbilical arterial/first postnatal blood from the infant, plus need for neonatal resuscitation for > 3 min.
3. Neurological signs of encephalopathy within 6 h of birth according to the NICHD classification for modified Sarnat staging [14].

Infants with congenital malformations, chromosomal abnormalities, metabolic disease, and evidence of intrauterine/perinatal infections with confirmed meningitis or with encephalopathy unrelated to birth asphyxia were excluded from the study. The control group were full-term infants who were born in the hospital in the same period

and were assayed for suspected infection but whose blood and CSF were found to be negative after culture; moreover, none had any findings that were suggestive of pathology in the brain.

Clinical assessments

All infants underwent neurological assessment shortly after birth before they were enrolled in the study. The assessment was then repeated approximately 12, 36, and 72 h and 7 days after birth and at discharge from the NICU. All assessments were conducted by the same neonatologist. HIE was classified as mild (HIE-I), moderate (HIE-II), or severe (HIE-III) according to the criteria of Sarnat and Sarnat [14].

All patients were treated under normothermic conditions and received standard treatment at the time of recruitment. This included fluid restriction, ionotropic support, and mechanical ventilation when needed as well as medical treatment for seizure activity. Continuous amplitude-integrated EEG was used to assess brain activity and suspected seizures. Given the clinical routine at the time of patient recruitment, all patients with moderate to severe encephalopathy underwent computed tomography (CT) brain scans and, in some cases, magnetic resonance imaging (MRI) on the third to fifth day of life.

All surviving patients were monitored with full neurological examinations at 3, 6, and 18 months of age that were conducted by an experienced neuropediatrician. The neurodevelopment of the patients who exhibited abnormal neurodevelopment or neurological signs on the examination at 18 months was assessed using the Bayley Scales of Infant and Toddler Development-II (BSID-II) [15], which was the Bayley version at that time. Outcome at 18 months was defined as normal outcome, adverse neurological outcome, or death. Adverse neurological outcome at 18 months was defined as a mental developmental index < 85, deafness, blindness, cerebral palsy, or seizure disorder.

The Hammersmith Infant Neurological Examination was performed on all control infants by an experienced neonatologist to get a standardized neurological assessment before discharge from NICU. Information on the outcome of the control infants at 18 months was gathered from outpatient pediatric care centers. All had normal neurological examination and none exhibited any abnormal neurological signs or history. None of the CSF samples from the controls showed indications of infection, and blood cultures were negative as well.

CSF analysis

CSF was collected from all patients within the first 3 days of life. CSF samples were stored at − 80 °C until analyzed. The CSF concentrations of IL-6, FasL, IL-6 receptor (IL-6R), and the soluble form of the Fas receptor (sFas) were measured by enzyme-linked immunosorbent assay (Diaclone Research, Besançon, France). The detection limits were 2 pg/mL (IL-6 and IL-6R), 12 pg/mL (FasL), and 47 pg/mL (sFas). The results were normalized against total protein content, which was measured using the bicinchoninic acid assay (Pierce, Rockford, IL, USA). In some patients and controls, competition enzyme immunoassays were used to measure prostaglandin E2 metabolite (PGEM). The assay was performed according to a commercial standardized protocol (Cayman Chemicals, Ann Arbor, MI, USA). Some of the PGEM data have been presented previously [16].

Statistical analyses

All clinical variables are presented as median (interquartile range). In terms of continuous variables, two independent groups were compared using the Mann-Whitney U test, while three independent groups were compared using the Kruskal-Wallis test with Dunn's multiple comparison post hoc test. Two related groups were compared using the Wilcoxon matched-pairs signed-rank test. Three related groups were compared using the Friedman test with Dunn's multiple comparison post hoc test. Correlations between variables were determined using the Spearman rank correlation test. In terms of categorical variables, the groups were compared using the chi-squared test. A graphical plot, namely, the receiver-operator characteristic (ROC) curve, was used to evaluate the ability of biomarkers to classify disease status and outcome. The maximum effectiveness of the biomarkers was evaluated using the Youdan Index. To evaluate the potential advantage of combining the FasL and IL-6 concentrations to identify degree of HIE and final outcome, we ranked the values of IL-6 and FasL. The rank sum was then subjected to ROC analysis.

All statistical tests were two-sided, and p values less than 0.05 were considered to indicate statistical significance.

Results

Patient characteristics at birth

In total, 46 term infants with HIE were initially enrolled into the study. Two infants with HIE who met the study eligibility criteria were then excluded because they were confirmed to have meningitis and suspected metabolic disease, respectively. Thus, 44 patients with HIE were finally included in the study. Twenty control infants without brain pathology who underwent LP for suspected infection but were then found to lack blood or CSF infection served as the control group.

The characteristics of the patient and control groups are summarized in Table 1. The two groups did not differ in terms of gestational age or birth weight. However, as expected, the HIE patients had significantly lower Apgar scores and blood gas values of pH than the control infants (both $p < 0.001$). Of the 44 patients, 14 (31.8%), 16 (36.4%),

Table 1 Clinical data of asphyxiated newborns and newborn nonasphyxiated controls

	Controls	HIE-I	HIE-II	HIE-III
Number of patients	20	14	16	14
Gestational age (week)[a]	39.5 (38.8 to 41.7)	41.1 (37.4 to 41.3)	40.1 (38.6 to 40.3)	39.0 (37.2 to 40.4)
Birth weight (g)[a]	3614 (2820 to 4570)	3500 (3240 to 4050)	3650 (3325 to 3975)	3310 (3225 to 3595)
Gender (female:male)[a]	10:10	7:7	7:9	8:6
1-min Apgar score**	9 (7 to 9)	2 (1 to 3)	1.5 (1 to 4)	1 (1 to 2)
5-min Apgar score**	10 (8 to 10)	5 (4 to 6)	3.5 (3 to 6)	3 (0 to 4)
10-min Apgar score**	10 (10 to 10)	6.5 (6 to 7)	5 (3 to 7)	4.5 (2 to 6)
Arterial pH**	7.40 (7.13 to 7.40)	7.00 (6.90 to 7.10)	7.00 (6.80 to 7.20)	6.90 (6.70 to 7.10)
BE[a]	− 2.5 (− 18.5 to − 0)	− 17.0 (− 19.3 to − 9.4)	− 24.0 (− 27.0 to − 17.0)	− 20.0 (− 25.0 to − 14.3)
Maternal infection[a]	0	2	2	2

Data are expressed as median (IQR)
[a]Not statistically significant
**$p < 0.0001$

and 14 (31.8%) were classified according to the classification system of Sarnat and Sarnat as having HIE-I, HIE-II, and HIE-III in the first days of life, respectively. The three HIE groups did not differ in terms of gestational age or birth weight.

Outcomes at 18 months

All control infants had normal outcomes at 18 months. The outcomes of the HIE patients at 18 months are summarized in Table 2. Thus, all 14 infants with HIE-I had a normal neurological examination at discharge from the NICU and a normal neurological outcome at 18 months. Of the 16 HIE-II infants, six had no neurological signs at discharge and were normal at the neurological assessment at 18 months. Two HIE-II patients had normal assessment at discharge but adverse neurological signs at 18 months. The remaining eight HIE-II patients had both neurological signs at discharge and abnormal neurological outcomes at 18 months. Thus, 10 patients with HIE-II had adverse neurological outcomes at 18 months. Of the 14 patients with HIE-III, eight died within the first 2 weeks of life of multiorgan failure due to asphyxia. The remaining six HIE-III patients all had both neurological signs at discharge and abnormal neurological outcomes at 18 months.

Table 2 Final outcome of asphyxiated newborns and newborn nonasphyxiated controls

	Controls	HIE-I	HIE-II	HIE-III
Number of patients	20	14	16	14
Outcome				
Normal	20	14	6	0
Adverse[a]	0	0	10	6
Death	0	0	0	8

HIE Hypoxic ischemic encephalopathy
[a]Adverse neurological outcome, including neurodevelopmental delay with developmental scores < 85 on BSID-III, deafness or blindness, and cerebral palsy or seizure disorder

Association between early CT/MRI findings and outcomes at 18 months

All 30 patients with HIE-II and HIE-III underwent CT and in some cases MRI on the third to fifth day after birth. All HIE-III infants and 8 of the 10 HIE-II infants who had an adverse neurological outcome at 18 months showed signs of edema on CT. In addition, three infants with HIE-II who had a normal neurological outcome at 18 months had signs of edema on CT. Four of the HIE-III infants also underwent MRI: the other patients did not undergo MRI because it was not routinely performed at the time of recruitment. All four HIE-III patients showed profound ischemic changes in the basal ganglia and thalami. Of these, two died in the neonatal period and one survived with adverse neurological outcomes at 18 months.

FasL and IL-6 levels in the CSF

In total, 76 CSF samples were gathered from the 44 patients and the 20 controls. One sample was obtained from 32 patients and 20 controls, and two samples were obtained from the remaining 12 patients. In the patients who underwent a single LP, the procedure was performed at a median of 22.5 (interquartile range, 15–42) h after birth and for controls 26 (13.5–48) h after birth. In the patients who underwent two LPs, the procedures were performed 14 (8–23) and 72 (60–111) h after birth, respectively. In the latter patients, the average FasL and IL-6 levels in the two CSF samples were used in the following statistical analyses.

The HIE patients had significantly higher FasL levels in the CSF (median, 62; interquartile range, 16–119 pg/mL) than the normal control infants (0, 0–0 pg/mL) ($p < 0.0001$). The patients with HIE-II (75.7, 43.7–129.4 pg/mL) and HIE-III (105.2, 28.5–168.6 pg/mL) also had significantly higher FasL levels than the patients with HIE-I (10.6, 0–41.6 pg/mL) and the controls (all $p < 0.0001$)

(Fig. 1a). Difference was not found between the HIE-II and HIE-III groups.

The HIE group also had significantly higher IL-6 levels in the CSF (77, 9–214 pg/mL) than the control infants (0, 0–12.4 pg/mL) ($p < 0.001$). The patients with HIE-II (37.7, 11.4–283 pg/mL) and HIE-III (179, 104–368 pg/mL) also had higher IL-6 levels than the patients with HIE-I (6.75, 0–50.2 pg/mL) and the controls (all $p < 0.0001$) (Fig. 1b).

Correlations between FasL and IL-6 levels with both HIE severity and outcome at 18 months

We assessed the relationship between FasL and IL-6 in the CSF and clinical outcomes. First, since Apgar score at 10 min predicts the neurological outcomes of infants with HIE [17], we assessed its relationship with the FasL and IL-6 levels in the CSF shortly after birth. FasL and IL-6 both correlated inversely with Apgar scores at 10 min ($r_s = -0.577$ and -0.622, respectively) (both $p < 0.0001$) (Additional file 1: Figure S1).

Second, correlation analyses showed that the CSF concentrations of FasL and IL-6 also correlated positively with the HIE grade ($r_s = 0.6898$ and 0.6864, respectively) (both $p < 0.0001$). Third, we assessed the relationship between clinical outcome at 18 months (normal or adverse neurological outcome or death) and the FasL and IL-6 levels in the CSF.

FasL

The patients with poor outcomes (i.e., adverse neurological outcome at 18 months or death) had higher FasL levels (105; 36.6–166 pg/mL) than the patients with favorable outcomes (10.6, 0–37.1 pg/mL) or the control infants (0, 0–0 pg/mL) (both $p < 0.0001$) (Fig. 2a). It should be noted, however, that the patients with adverse outcomes varied markedly in their FasL levels. Indeed, two of the patients who died had FasL levels below the detection limit. The patients who survived with adverse neurological outcomes did not differ from the patients who died in terms of CSF FasL levels. Correlation analysis showed that the CSF concentrations of FasL correlated positively with poor 18-month clinical outcomes ($r_s = 0.7017$) ($p < 0.0001$).

IL-6

The patients with poor outcomes had significantly higher IL-6 levels (162, 35.6–264 pg/mL) than the patients with normal outcomes (9.35, 0–40.8 pg/mL) or the control infants (0, 0–5.35 pg/mL) (both $p < 0.0001$) (Fig. 2b). The patients who survived with adverse neurological outcome did not differ from the patients who died in terms of CSF IL-6 levels. Correlation analyses showed that the CSF concentrations of IL-6 correlated positively with poor 18-month clinical outcomes ($r_s = 0.7017$) ($p < 0.0001$).

Time-dependent trends in FasL and IL-6 levels in the CSF

Twelve patients provided a CSF sample at two different time points. All had HIE-II or HIE-III. Nine had adverse outcomes at 18 months. The remaining three had normal outcomes. In all patients, the FasL levels were higher in the second sample ($p = 0.0025$) (Fig. 3a).

In relation to IL-6, the levels were lower in the second sample in all but three cases ($p = 0.0522$) (Fig. 3b). In these three cases, the patients had low IL-6 levels in the first sample. Two of these three patients had normal outcomes. The nine patients who exhibited lower IL-6 levels in the second sample all had adverse 18-month outcomes. Thus, over time, FasL and IL-6 levels in the CSF rose and fell, respectively.

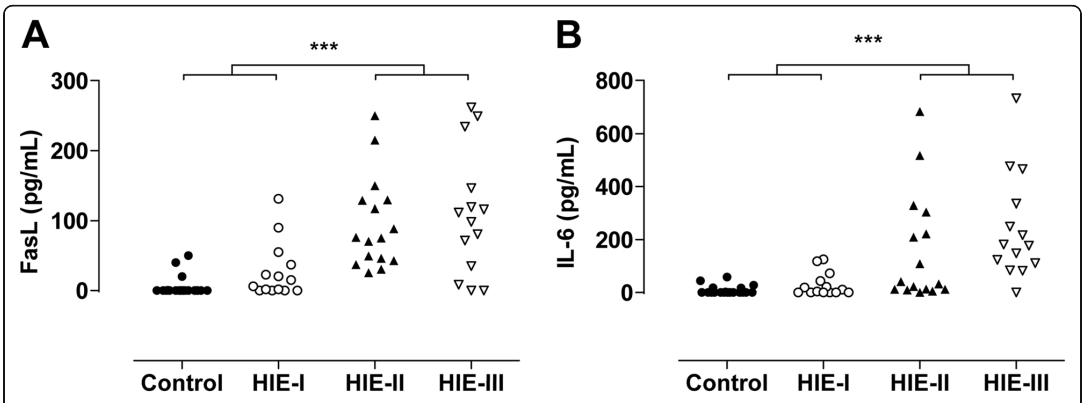

Fig. 1 Fas-ligand (FasL) and interleukin-6 (IL-6) levels in the cerebrospinal fluid (CSF) correlate with the degree of hypoxic-ischemic encephalopathy (HIE), which was classified as mild (HIE-I, $n = 14$), moderate (HIE-II, $n = 16$), or severe (HIE-III, $n = 14$). The controls were infants with no signs of HIE ($n = 20$). FasL levels (pg/mL) (**a**) and IL-6 levels (pg/mL) (**b**) were higher in the patients with HIE-III and HIE-II than in the patients with HIE-I and the controls. ***$p < 0.0001$

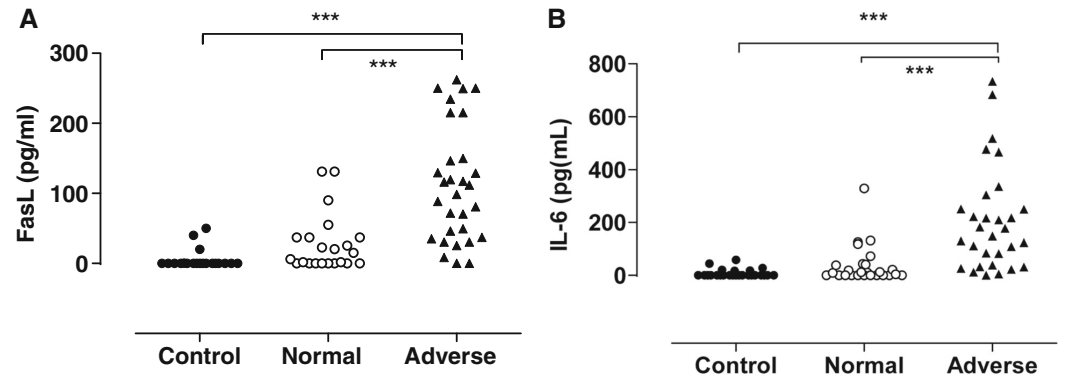

Fig. 2 Fas-ligand (FasL) and interleukin-6 (IL-6) levels in the cerebrospinal fluid (CSF) correlate with the long-term outcomes after perinatal asphyxia. Adverse outcome is defined as adverse neurological outcome at the 18-month assessment or death. FasL levels (pg/mL) (**a**) and IL-6 (pg/mL) levels (**b**) were higher in patients with an adverse outcome ($n = 24$) than in patients with a normal outcome ($n = 20$) and the controls ($n = 20$). ***$p < 0.0001$

FasL and IL-6 levels in the CSF are indicators for HIE severity

ROC curves for FasL and IL-6 levels in relation to HIE were generated, and the areas under the ROC curves (AUCs) were estimated. These analyses showed that FasL and IL-6 predicted HIE with AUC values of 0.89 and 0.87, respectively (Fig. 4a, b). The Youdan Indices were then calculated: thus, the FasL cutoff of > 24 pg/mL predicted HIE with the highest sensitivity (90%) and specificity (82.4%), and the IL-6 cutoff of > 77 pg/mL predicted HIE with the highest sensitivity (66.7%) and specificity (94.1%).

Combining the IL-6 and FasL using rank order rendered a positive predictive value of 0.94. The sensitivity was 86.7% and specificity 91.2% in relation to degree of HIE (Fig. 4c). Notably, using the two cutoff values together also predicted HIE grade II–III with a sensitivity of 100% and a specificity of 79.4%, Table 3.

To determine how well FasL and IL-6 predict HIE, we also assessed the ability of cord blood pH, which is the established biomarker for perinatal asphyxia, to predict HIE. Cord blood pH predicted HIE with an AUC of 0.80. Cord blood pH < 6.85 predicted HIE with a sensitivity of 66.7% and a specificity of 80.0% (data not

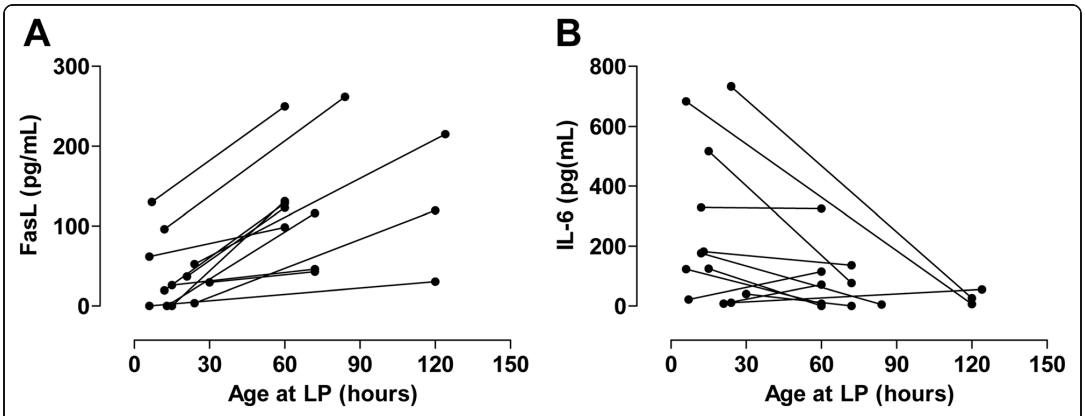

Fig. 3 Kinetics of Fas-ligand (FasL) and interleukin-6 (IL-6) in the cerebrospinal fluid (CSF) from the 12 patients who provided a sample at two different time points. Lines indicate the samples from the same patient. **a** All patients had higher FasL levels in the later samples. **b** In 9 of the 12 patients, the IL-6 levels were higher in the earlier samples

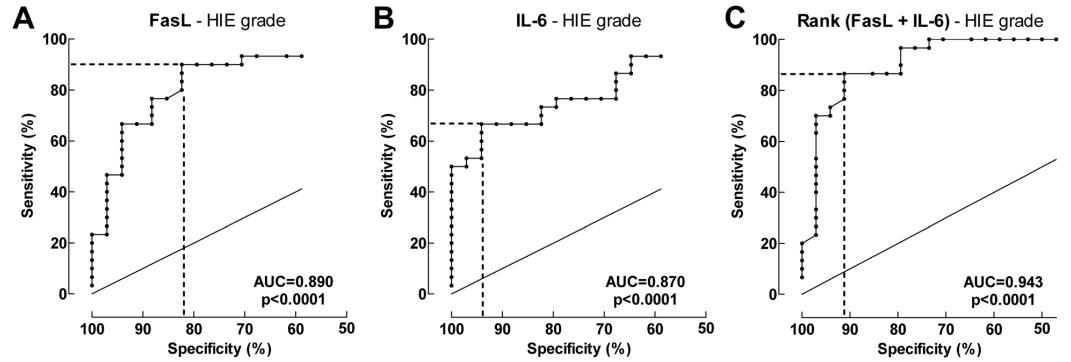

Fig. 4 Receiver-operator characteristic (ROC) curve of Fas-ligand (FasL) and interleukin-6 (IL-6) in relation to hypoxic-ischemic encephalopathy (HIE) grade. Control and HIE I versus HIE grade II–III. **a** The AUC for FasL was 0.89. The FasL cutoff value of 24 pg/mL (dashed lines) predicted HIE grade with a sensitivity of 90.0% and a specificity of 82.4%. **b** The AUC for IL-6 was 0.87. At the cutoff value of 77 pg/mL (dashed lines), IL-6 predicted HIE grade with a sensitivity of 66.7% and a specificity of 94.1%. **c** Using a combination of ranks for IL-6 and Fas-L, the sensitivity was 86.7% and specificity 91.2% in relation to degree of HIE. The sum for ranking of IL-6 and Fas-L was used for the evaluation. The AUC for the sum for ranking was 0.94

shown). Thus, FasL and IL-6 alone and together predicted the degree of HIE better than this well-known standard marker of perinatal asphyxia.

Ability of FasL and IL-6 levels in the CSF to predict adverse outcomes at 18 months

ROC curves for FasL and IL-6 levels in relation to the outcomes at 18 months were generated, and the AUCs were estimated. These analyses showed that FasL and IL-6 predicted adverse 18-month outcomes (i.e., adverse neurological outcome or death) with AUCs of 0.86 and 0.90, respectively (Fig. 5a, b). The Youdan Indices were calculated: the FasL cutoff of > 45 pg/mL predicted adverse outcomes with a sensitivity of 79.2% and a specificity of 82.5%, and the IL-6 cutoff of > 77 pg/mL predicted adverse outcomes with a sensitivity of 79.2% and a specificity of 92.5%.

Combining the IL-6 and FasL using rank order rendered a positive predictive value of 0.94**.** The sensitivity was 95.8% and specificity 85.7% in relation to outcome (Fig. 5c). Notably, using the two cutoff values together also predicted adverse outcome with a sensitivity of 100% and a specificity of 80.0% (Table 3).

By contrast, cord blood pH predicted adverse outcomes with an AUC of 0.86. Cord blood pH < 6.85 predicted adverse outcomes with a sensitivity of 66.7% and a specificity of 93.6% (data not shown). Thus, FasL and IL-6 alone and together predicted adverse outcomes at

Table 3 Sensitivity and specificity of outcome of asphyxiated newborns and newborn nonasphyxiated controls

	HIE grade			Outcome		
	IL-6 > 77 pg/mL	FasL > 24 pg/mL	IL-6 > 77 pg/mL and/or FasL > 24 pg/mL	IL-6 > 77 pg/mL	FasL > 45 pg/mL	IL6 > 77 pg/mL and/or FasL > 45 pg/mL
True positive	20	27	30	19	19	24
True negative	32	28	27	37	33	32
False positive	2	6	7	3	7	8
False negative	10	3	0	5	5	0
Specificity (%)	94.1	82.4	79.4	92.5	82.5	80.0
Sensitivity (%)	66.7	90.0	100.0	79.2	79.2	100.0
Accuracy (%)	81.3	85.9	89.1	87.5	81.3	87.5
Negative predictive values (%)	76.2	90.3	100.0	88.1	86.8	100.0
Positive predictive values (%)	90.9	81.8	81.1	86.4	73.1	75.0

HIE Hypoxic ischemic encephalopathy
[a]Death or Adverse neurological outcome, including delayed neurodevelopment with mental developmental index (MDI) < 85, deafness or blindness, and cerebral palsy or seizure disorder

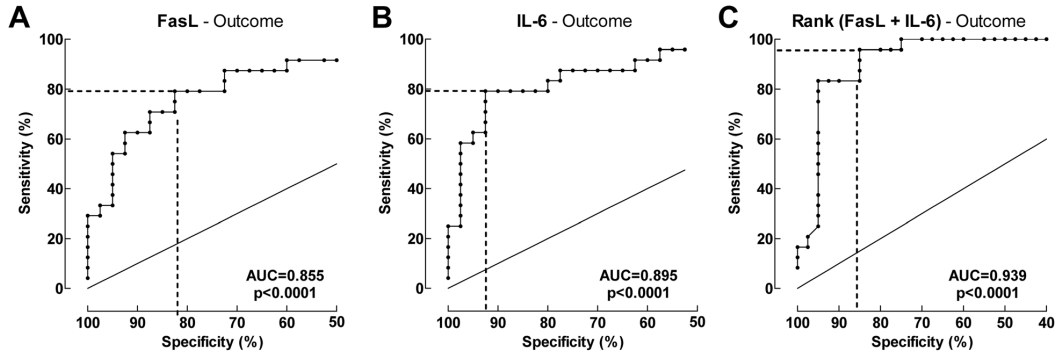

Fig. 5 Receiver-operator characteristic (ROC) curve of Fas-ligand (FasL) and interleukin-6 (IL-6) in relation to adverse outcome. Adverse outcome is defined as adverse neurological outcome at the 18-month assessment or death. **a** The AUC for FasL was 0.86. The FasL cutoff value of 45 pg/mL (dashed lines) predicted adverse outcomes with a sensitivity of 79.2% and a specificity of 82.5%. **b** The AUC for IL-6 was 0.90. The IL-6 cutoff value of 77 pg/mL (dashed lines) predicted adverse outcomes with a sensitivity of 79.2% and a specificity of 92.5%. **c** Using a combination of ranks for IL-6 and Fas-L, the sensitivity was 95.8% and specificity 85.7% in relation to outcome. The sum for ranking of IL-6 and Fas-L was used for the evaluation. The AUC for the sum for ranking was 0.94

18 months better than this well-known standard marker of perinatal asphyxia.

Soluble forms of FasL and IL-6 receptors in the CSF

Our findings prompted us to determine the levels of the soluble forms of the receptors for FasL and IL-6. sFas levels were measured in 26 patient samples and 20 control samples. Seven patient samples had high sFas levels (ranging from 355 to 1160 pg/mL), and all but one of these patients had an adverse outcome at 18 months. The median sFas concentration of the patients was 441 pg/mL. By contrast, sFas levels in all control infant samples were below the limits of detection (Additional file 2: Table S1).

All patient CSF samples had significant amounts (i.e., above the upper limit of the test) of IL-6R. By contrast, the IL-6R levels in all control infant samples were below the limits of detection (data not shown).

Relationship between PGEM and IL-6 levels in the CSF

We reported previously that increased levels of PGEM in the CSF correlate with 18-month clinical outcomes of infants with HIE [16]. We measured the PGEM levels in the CSF of 25 patients and nine controls in this study. We found that the PGEM levels correlated significantly with the IL-6 levels in the CSF ($p = 0.0009$) (Fig. 6). In contrast, no correlation was found between the FasL levels and the PGEM levels.

The inclusion of PGEM data in addition to IL-6 and FasL data in Table 3 increased neither specificity nor sensitivity for identification of patients with HIE grade II–III or poor outcome.

Discussion

The main findings of this study were that post-asphyxiated neonates had elevated levels of the inflammatory mediators IL-6 and FasL in their CSF shortly after birth. Since the levels of both molecules correlated positively with HIE grade and poor 18-month clinical outcome, they may be useful as biomarkers for the severity of hypoxic brain injury and for predicting the long-term outcome after perinatal asphyxia.

Animal and human studies show that a variety of cytokines are expressed in the brain in cerebral ischemia and that the cytokine profile associates significantly with the severity of ischemic brain damage [18, 19]. Moreover, it has been suggested that cytokines can either induce or ameliorate ischemic brain injury and that these opposing

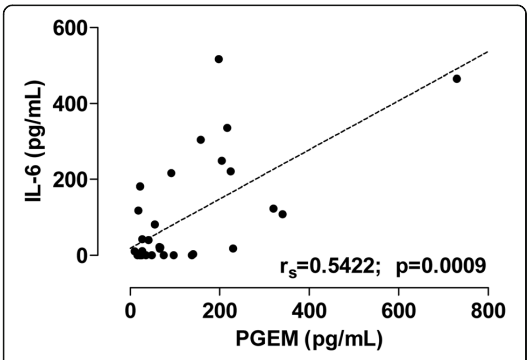

Fig. 6 Levels of interleukin-6 (IL-6) and the prostaglandin E2 metabolite (PGEM) in the cerebrospinal fluid (CSF) correlate ($p = 0.0009$). r_s Spearman rank correlation coefficient

functions depend on both the phase of the neural cell damage and the severity of the insult [10]. These time- and severity-dependent changes in cytokine levels mean that the levels of a given cytokine in a CSF sample will only provide a snapshot of an ongoing process; it is often difficult to know when exactly a cytokine is promoting ischemic brain injury or acting protectively. This is exemplified by several studies on the role of IL-6 in therapeutic hypothermia, which is thought to be neuroprotective because it has anti-inflammatory properties [20]. When Jenkins and colleagues serially measured cytokines every 12 h for 4 days after the birth, they found that HIE patients who underwent hypothermia treatment had higher serum IL-6 levels at all time points than HIE infants who were treated under normothermic conditions [13]. Moreover, the IL-6 levels in the hypothermia-treated group were biphasic while the IL-6 levels in the normothermic patients tended to decline over time. Significantly, while high IL-6 levels early after the insult, in both groups, are associated with adverse 18-month outcomes, a secondary peak of IL-6 is associated with better outcomes [13, 21]. This suggests that IL-6 may have biphasic roles in the pathogenesis of hypoxic brain injury: it induces inflammation and injury early after the insult but then contributes to cytokine-mediated repair at later time points [21, 22].

In the present study, the patients did not receive therapeutic hypothermia because it was not an established therapy for HIE at the time of recruitment. We found that, in the 12 patients who underwent LP at two separate time points after birth, the IL-6 levels were lower in the second sample in all but three cases (Fig. 3b). This is consistent with previous observations, regarding normothermic patients where serum IL-6 levels after birth asphyxia were characterized in [13, 21]. All patients in the present study who exhibited a drop in IL-6 levels had adverse 18-month outcomes. Two of the three patients who had increased IL-6 levels between first and second LP had normal outcomes. However, those patients had low IL-6 levels in the first sample and the rise was small (9.9 pg/mL, 9–10.5 in LP1 and 63.3 pg/mL, 59.5–67 in LP2) (Fig. 3b).

It should be noted that we measured IL-6 levels in the CSF, whereas several studies measure the serum IL-6 levels, e.g., [13, 21]. The fact that our findings closely resemble those of normothermic patients [13] probably reflects the fact that the serum and CSF concentrations of IL-6 in term infants with asphyxia correlate [23].

A systematic review of potential brain injury biomarkers identified serum IL-6 as one of the few independent predictors of adverse outcome in survivors of HIE [24]. However, CSF IL-6 may be a better predictor of adverse outcomes because local cytokine profiles are often poorly reflected in the plasma: this is because the plasma levels can also be shaped by secondary reactions to the injury [25, 26].

Intracellular cytokine levels, which closely reflect cytokine production at a cellular level and show more stable kinetics in time, have recently been analyzed in term infants requiring systemic hypothermia after perinatal asphyxia [27]. In that study, intracellular IL-6 levels in CD4+ peripheral blood mononuclear cells peaked at 24 h post-asphyxia. However, IL-6 levels exhibited a large variability and did not differ between the two patient groups moderate ($n = 17$) and severe ($n = 11$) asphyxia. Thus, IL-6 plays an important role in the initial inflammatory response, but determination of CSF levels of IL-6 might better predict adverse outcomes in survivors of HIE [28].

IL-6 initiates a signal transduction cascade by binding to specific IL-6 membrane receptors (IL-6R). Apoptosis associates with the shedding of membrane components, including IL-6R, which then facilitates IL-6 signaling in neighboring cells [29]. While most brain cells are not responsive to IL-6 alone, they can be stimulated by IL-6 bound to a soluble form of IL-6 receptor in a process called trans-signal activation [30]. The present study showed that the HIE patients had high concentrations of IL-6R in all samples whereas the IL-6R levels in all control infants were below the detection limit. It is possible that the high levels of IL-6R in the CSF of the HIE patients reflect an apoptotic process that is taking place in the brain at the time the samples were gathered. Unfortunately, the CSF samples were limited in volume, which meant that the IL-6R measurements were not accurate enough to assess the relationship between IL-6R levels and long-term outcomes.

This study showed that FasL, which plays a key role in apoptosis, also correlated positively with both the HIE grade (Fig. 1a) and the adverse long-term outcomes (Fig. 2a) in HIE patients. Experimental studies suggest that hypothermia blocks the Fas-mediated intrinsic and extrinsic apoptosis pathways [31, 32]; however, clinical studies are needed to confirm this. The naturally occurring soluble form of Fas receptor, sFas, prevents cell ligation with FasL, and this blocking of FasL inhibits neuronal cell apoptosis in experimental brain ischemia [33]. sFas has been found in the CSF from neonates with hydrocephalus [34], but in that study, FasL was not detected. We found that, while none of the control infants had sFas in their CSF, 7 of 26 HIE patients had high levels and that all but one of these had adverse outcomes at 18 months. This suggests that CSF sFas may not actually protect HIE infants from FasL-induced apoptosis and the consequent poor outcomes.

Prostaglandin E2 (PGE2) is another important mediator of neuroinflammation [35]. PGE2 and its derivative PGEM rapidly increase during hypoxia [36]. We found

that IL-6 levels in the CSF correlated positively with the PGEM levels in the CSF (Fig. 6). However, since only a subgroup of the patient cohort was examined for both PGEM and IL-6, and we have already established that increased levels of PGEM in CSF correlate positively with the clinical outcome of infants with HIE [16], we thus focused our further analyses on FasL and IL-6.

Our ROC curve analyses showed that IL-6 and FasL in the CSF may be useful as additional tools for evaluating the severity of hypoxic-ischemic encephalopathy in post-asphyxiated infants and for predicting the long-term outcomes. First, this study showed that FasL (AUC = 0.89) and IL-6 (AUC = 0.87) predict HIE grade even better than cord blood pH (AUC = 0.80). When FasL and IL-6 were combined, they predicted HIE grade particularly well (AUC = 0.94). Second, FasL (AUC = 0.90) and IL-6 (AUC = 0.86) predicted adverse outcomes better than cord blood pH (0.86). This performance was even better when FasL and IL-6 were combined (AUC = 0.94).

Notably, this novel and unprecedented results of prediction for HIE and long-term outcome, using CSF biomarkers, could help guide treatment decisions. Thus, prospective studies concerning the benefits of CSF cytokine measurements also in hypothermic treated patients would indeed be of value.

The main limitation of this study was the time lapse between patient recruitment and the presentation of the results. However, the fact that the patients were recruited before hypothermia became the standard treatment for HIE could be considered an advantage because it allowed us to compare our FasL and IL-6 findings in that setting with the findings of other studies, which were all conducted after therapeutic hypothermia was introduced as a standard treatment. Another limitation is that, while an experienced pediatric neurologist performed the routine clinical follow-up on all post-asphyxiated children at the Karolinska Hospital at that time, only the patients with neurological symptoms or signs at 18 months were assessed with the Bayley developmental evaluation assessment: the patients without signs did not undergo BSID-II scoring. Further studies on the diagnostic benefits of monitoring FasL and IL-6, with or without other biomarkers, in newborn infants with HIE who are treated with therapeutic hypothermia are warranted. Further studies on these biomarkers may also shed more light on the pathology of brain damage and determine whether targeting them may be of therapeutic benefit.

Conclusion

IL-6 and FasL are released into the CSF during birth asphyxia. These biomarkers support the clinical diagnosis of HIE degree, thus correlating to the degree of brain injury, and are useful for predicting the long-term clinical outcome. Thus, they may aid the early decision-making regarding treatment of asphyxiated newborns.

Additional files

Additional file 1: Figure S1. Fas-ligand (FasL) (A) and Interleukin-6 (IL-6) (B) levels in the cerebrospinal fluid (CSF) correlate inversely with Apgar scores at 10 min ($r_s = -0.577$ and -0.622, respectively. r_s = Spearman rank correlation coefficient) (both $p < 0.0001$). (TIF 119 kb)

Additional file 2: Table S1. Soluble Fas receptor (sFas) levels in 26 asphyxia patients and 20 controls. (DOCX 15 kb)

Abbreviations

AUC: Area Under the ROC curve; BSID-II: Bayley Scales of Infant and Toddler Development-II; CSF: Cerebrospinal fluid; CT: Computed tomography; FasL: Fas-ligand; HIE: Hypoxic-ischemic encephalopathy; IL-6: Interleukin (IL)-6; IL-6R: IL-6 receptor; LP: Lumbar puncture; MRI: Magnetic resonance imaging; PGE2: Prostaglandin E2; PGEM: Prostaglandin E2 metabolite; ROC: Receiver-operator characteristic; sFas: Soluble form of Fas receptor

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

KL was responsible for the concept and study design, recruited patients, performed immunoassays, analyzed and interpreted the patient data, wrote the first draft, and was a major contributor in writing the manuscript. HM was involved in the study design and data analysis. SE was a major contributor to the statistical analyses of the data and revised the manuscript. EH was involved in designing the study, recruited patients, analyzed and interpreted the patient data, funded the study, and was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was performed in accordance with the tenets of the declaration of Helsinki 1975 and its revision in 1983 and European Community guidelines. The regional ethics committees at the Karolinska Institutet and the Stockholm County approved the study (Dnr 98-246, 2003-174, 2011/1891-31). Informed written consent was obtained from the parents of the enrolled patients.

Consent for publication

Not applicable

Competing interests

Dr. Herlenius is employed by the Karolinska University Hospital and the Karolinska Institutet and is also co-inventor of a patent application regarding biomarkers and their relation to breathing disorders (Patent Application No.

WO2009063226). None of the other authors have a conflict of interest related to the study.

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References

1. Graham EM, Ruis KA, Hartman AL, Northington FJ, Fox HE. A systematic review of the role of intrapartum hypoxia-ischemia in the causation of neonatal encephalopathy. Am J Obstet Gynecol. 2008;199:587–95.
2. Rainaldi MA, Perlman JM. Pathophysiology of birth asphyxia. Clin Perinatol. 2016;43:409–22.
3. Tagin MA, Woolcott CG, Vincer MJ, Whyte RK, Stinson DA. Hypothermia for neonatal hypoxic ischemic encephalopathy: an updated systematic review and meta-analysis. Arch Pediatr Adolesc Med. 2012;166:558–66.
4. Broughton BR, Reutens DC, Sobey CG. Apoptotic mechanisms after cerebral ischemia. Stroke. 2009;40:e331–9.
5. Graham EM, Sheldon RA, Flock DL, Ferriero DM, Martin LJ, O'Riordan DP, Northington FJ. Neonatal mice lacking functional Fas death receptors are resistant to hypoxic-ischemic brain injury. Neurobiol Dis. 2004;17:89–98.
6. Lenzlinger PM, Marx A, Trentz O, Kossmann T, Morganti-Kossmann MC. Prolonged intrathecal release of soluble Fas following severe traumatic brain injury in humans. J Neuroimmunol. 2002;122:167–74.
7. Schmitz T, Felderhoff-Mueser U, Sifringer M, Groenendaal F, Kampmann S, Heep A. Expression of soluble Fas in the cerebrospinal fluid of preterm infants with posthemorrhagic hydrocephalus and cystic white matter damage. J Perinat Med. 2011;39:83–8.
8. Stridh L, Mottahedin A, Johansson ME, Valdez RC, Northington F, Wang X, Mallard C. Toll-like receptor-3 activation increases the vulnerability of the neonatal brain to hypoxia-ischemia. J Neurosci. 2013;33:12041–51.
9. Fang XX, Jiang XL, Han XH, Peng YP, Qiu YH. Neuroprotection of interleukin-6 against NMDA-induced neurotoxicity is mediated by JAK/STAT3, MAPK/ERK, and PI3K/AKT signaling pathways. Cell Mol Neurobiol. 2013;33:241–51.
10. Suzuki S, Tanaka K, Suzuki N. Ambivalent aspects of interleukin-6 in cerebral ischemia: inflammatory versus neurotrophic aspects. J Cereb Blood Flow Metab. 2009;29:464–79.
11. Savman K, Blennow M, Gustafson K, Tarkowski E, Hagberg H. Cytokine response in cerebrospinal fluid after birth asphyxia. Pediatr Res. 1998;43:746–51.
12. Orrock JE, Panchapakesan K, Vezina G, Chang T, Harris K, Wang Y, Knoblach S, Massaro AN. Association of brain injury and neonatal cytokine response during therapeutic hypothermia in newborns with hypoxic-ischemic encephalopathy. Pediatr Res. 2016;79:742–7.
13. Jenkins DD, Rollins LG, Perkel JK, Wagner CL, Katikaneni LP, Bass WT, Kaufman DA, Horgan MJ, Languani S, Givelichian L, et al. Serum cytokines in a clinical trial of hypothermia for neonatal hypoxic-ischemic encephalopathy. J Cereb Blood Flow Metab. 2012;32:1888–96.
14. Sarnat HB, Sarnat MS. Neonatal encephalopathy following fetal distress. A clinical and electroencephalographic study. Arch Neurol. 1976;33:696–705.
15. Cirelli I, Bickle Graz M, Tolsa JF. Comparison of Griffiths-II and Bayley-II tests for the developmental assessment of high-risk infants. Infant Behav Dev. 2015;41:17–25.
16. Bjork L, Leifsdottir K, Saha S, Herlenius E. PGE-metabolite levels in CSF correlate to HIE score and outcome after perinatal asphyxia. Acta Paediatr. 2013;102(11):1041–7.
17. Natarajan G, Shankaran S, Laptook AR, Pappas A, Bann CM, McDonald SA, Das A, Higgins RD, Hintz SR, Vohr BR, et al. Apgar scores at 10 min and outcomes at 6–7 years following hypoxic-ischaemic encephalopathy. Arch Dis Child Fetal Neonatal Ed. 2013;98:F473–9.
18. Chalak LF, Sanchez PJ, Adams-Huet B, Laptook AR, Heyne RJ, Rosenfeld CR. Biomarkers for severity of neonatal hypoxic-ischemic encephalopathy and outcomes in newborns receiving hypothermia therapy. J Pediatr. 2014;164:468–474 e461.
19. Wang H, Guo W, Liu H, Zeng R, Lu M, Chen Z, Xiao Q. Inhibition of inflammatory mediator release from microglia can treat ischemic/hypoxic brain injury. Neural Regen Res. 2013;8(13):1157–68.
20. Diestel A, Roessler J, Berger F, Schmitt KR. Hypothermia downregulates inflammation but enhances IL-6 secretion by stimulated endothelial cells. Cryobiology. 2008;57:216–22.
21. Roka A, Beko G, Halasz J, Toldi G, Lakatos P, Azzopardi D, Tulassay T, Szabo M. Changes in serum cytokine and cortisol levels in normothermic and hypothermic term neonates after perinatal asphyxia. Inflamm Res. 2013;62:81–7.
22. Liu S, Zhu S, Zou Y, Wang T, Fu X. Knockdown of IL-1beta improves hypoxia-ischemia brain associated with IL-6 up-regulation in cell and animal models. Mol Neurobiol. 2015;51:743–52.
23. Tekgul H, Yalaz M, Kutukculer N, Ozbek S, Kose T, Akisu M, Kultursay N, Gokben S. Value of biochemical markers for outcome in term infants with asphyxia. Pediatr Neurol. 2004;31:326–32.
24. Ramaswamy V, Horton J, Vandermeer B, Buscemi N, Miller S, Yager J. Systematic review of biomarkers of brain injury in term neonatal encephalopathy. Pediatr Neurol. 2009;40:215–26.
25. Aly H, Khashaba MT, El-Ayouty M, El-Sayed O, Hasanein BM. IL-1beta, IL-6 and TNF-alpha and outcomes of neonatal hypoxic ischemic encephalopathy. Brain and Development. 2006;28:178–82.
26. Silveira RC, Procianoy RS. Interleukin-6 and tumor necrosis factor-alpha levels in plasma and cerebrospinal fluid of term newborn infants with hypoxic-ischemic encephalopathy. J Pediatr. 2003;143:625–9.
27. Bajnok A, Berta L, Orban C, Veres G, Zadori D, Barta H, Meder U, Vecsei L, Tulassay T, Szabo M, Toldi G. Distinct cytokine patterns may regulate the severity of neonatal asphyxia-an observational study. J Neuroinflammation. 2017;14:244.
28. Hagberg H, Mallard C, Ferriero DM, Vannucci SJ, Levison SW, Vexler ZS, Gressens P. The role of inflammation in perinatal brain injury. Nat Rev Neurol. 2015;11:192–208.
29. Chalaris A, Rabe B, Paliga K, Lange H, Laskay T, Fielding CA, Jones SA, Rose-John S, Scheller J. Apoptosis is a natural stimulus of IL6R shedding and contributes to the proinflammatory trans-signaling function of neutrophils. Blood. 2007;110:1748–55.
30. Jung JE, Kim GS, Chan PH. Neuroprotection by interleukin-6 is mediated by signal transducer and activator of transcription 3 and antioxidative signaling in ischemic stroke. Stroke. 2011;42:3574–9.
31. Drury PP, Bennet L, Gunn AJ. Mechanisms of hypothermic neuroprotection. Semin Fetal Neonatal Med. 2010;15:287–92.
32. Liu X, Wang M, Chen H, Guo Y, Ma F, Shi F, Bi Y, Li Y. Hypothermia protects the brain from transient global ischemia/reperfusion by attenuating endoplasmic reticulum response-induced apoptosis through CHOP. PLoS One. 2013;8:e53431.
33. Chen B, Wu Z, Xu J, Xu Y. Calreticulin binds to Fas ligand and inhibits neuronal cell apoptosis induced by ischemia-reperfusion injury. Biomed Res Int. 2015;2015:895284.
34. Felderhoff-Mueser U, Buhrer C, Groneck P, Obladen M, Bartmann P, Heep A. Soluble Fas (CD95/Apo-1), soluble Fas ligand, and activated caspase 3 in the cerebrospinal fluid of infants with posthemorrhagic and nonhemorrhagic hydrocephalus. Pediatr Res. 2003;54:659–64.
35. Siljehav V, Olsson Hofstetter A, Jakobsson PJ, Herlenius E. mPGES-1 and prostaglandin E2: vital role in inflammation, hypoxic response, and survival. Pediatr Res. 2012;72:460–7.
36. Siljehav V, Hofstetter AM, Leifsdottir K, Herlenius E. Prostaglandin E2 mediates cardiorespiratory disturbances during infection in neonates. J Pediatr. 2015;167:1207–13. e1203

Paper IV

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ACTA PÆDIATRICA NURTURING THE CHILD WILEY

ORIGINAL ARTICLE

Proteomic profiles in cerebrospinal fluid predicted death and disability in term infants with perinatal asphyxia: A pilot study

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Abstract

Aim: Perinatal asphyxia, resulting in hypoxic-ischaemic encephalopathy (HIE), has been associated with high mortality rates and severe lifelong neurodevelopmental disabilities. Our aim was to study the association between the proteomic profile in cerebrospinal fluid (CSF) and the degree of HIE and long-term outcomes.

Methods: We prospectively enrolled 18-term born infants with HIE and 10-term born controls between 2000 and 2004 from the Karolinska University Hospital. An antibody suspension bead array and FlexMap3D analysis was used to characterise 178 unique brain-derived and inflammation associated proteins in their CSF.

Results: Increased CSF concentrations of several brain-specific proteins were observed in the proteome of HIE patients compared with the controls. An upregulation of neuroinflammatory pathways was also noted and this was confirmed by pathway analysis. Principal component analysis revealed a gradient from favourable to unfavourable HIE grades and outcomes. The proteins that provided strong predictors were structural proteins, including myelin basic protein and alpha-II spectrin. The functional proteins included energy-related proteins like neuron-specific enolase and synaptic regulatory proteins. Increased CSF levels of 51 proteins correlated with adverse outcomes in infants with HIE.

Conclusion: Brain-specific proteins and neuroinflammatory mediators in CSF may predict HIE degrees and outcomes after perinatal asphyxia.

KEYWORDS
biomarkers, cerebrospinal fluid, hypoxic-ischaemic encephalopathy, perinatal asphyxia, protein profile

1 | INTRODUCTION

Four million infants experience perinatal asphyxia, leading to hypoxic-ischaemic encephalopathy (HIE), each year. HIE is one of the most common contributors to early neonatal mortality.[1] The incidence of moderate to severe HIE is 1–3 per 1,000 live births in high-income countries.[2] Hypoxic-ischaemic brain damage is a complex process that represents an evolving cascade of harmful events. The

Abbreviations: CSF, cerebrospinal fluid; HIE, hypoxic-ischaemic encephalopathy; IQR, interquartile range.

primary phase of the injury, during exposure to hypoxic ischaemia, is followed by a latent phase, where the cerebral oxidative metabolism may partially or completely recover. Therapeutic hypothermia is the only treatment that is available for moderate to severe HIE. It is applied during the latent phase, to ameliorate the secondary phase of progressive energy failure and brain cell death.[3] However, the neuroprotective effect of therapeutic hypothermia is limited. The mortality rate is still high and infants that survive may face lifelong disabilities, including cerebral palsy, epilepsy and cognitive impairment. Accumulating evidence indicates that inflammation contributes to a prolonged hypoxic-ischaemic brain injury, which may last for months or even years. This, in turn, provides the potential for adjunctive treatment options at later stages.[4]

Reliable biomarkers that reflect the complex pathology of HIE could facilitate the evolution of targeted neuroprotective treatment approaches and provide early identification of the patients that could be at risk of long-term sequelae. It has been suggested that various biomarkers may be useful when it comes to predicting outcomes of neonatal HIE, but none of these have been established in clinical settings.[5] Affinity-based proteomic techniques offer a novel insight into the underlying pathophysiology of brain disease. It enables large numbers of proteins to be simultaneously analysed in small samples. Protein arrays have been used in preclinical and clinical studies of adults with traumatic brain injuiries.[6,7] They have also been used for protein profiling of cerebrospinal fluid (CSF) from preterm infants.[8] The present study used antibody suspension bead array technology to assess the levels of brain enriched proteins and known inflammatory mediators[9] in the CSF of infants with HIE. We then compared these results with non-asphyxiated infants, who formed the control group.

The study had two aims. First, we aimed to evaluate the use of protein arrays in predicting long-term outcomes following perinatal asphyxia. Secondly, we wanted to discover novel biomarkers for bedside use when treating these patients.

Key notes

- Cerebrospinal fluid (CSF) proteomes following perinatal asphyxia provide valuable information on the pathogenesis and prognosis of brain injuries.
- This study identified a proteomic profile in CSF following perinatal asphyxia, representing upregulation of neuroinflammatory pathways and various pathological cascades of hypoxic brain injuries.
- Several of these novel proteins correlated with the degree of hypoxic-ischaemic encephalopathy (HIE) and unfavourable outcomes and had a diagnostic and predictive value for HIE.

2 | PATIENTS AND METHODS

2.1 | Study population

We prospectively enrolled 18 term-born infants with perinatal asphyxia from the neonatal intensive care unit at the Karolinska University Hospital in Stockholm, Sweden, between October 2000 and September 2004. The controls were 10 term-born infants without a history of asphyxia from the Hospital's general medical neonatal ward. The infants were included in the asphyxia group if they had undergone clinically indicated lumbar punctures and fulfilled the criteria for perinatal asphyxia, by showing signs of foetal and postnatal distress. These included foetal bradycardia or decelerations on cardiotocographic registration and a pH of <7.1 or a lactate of >4.8 in scalp blood. The Apgar score needed to be under 6 at 5 min and their umbilical arterial blood, or blood collected within an hour of birth, needed to have a pH of ≤7.00 and/or a base deficit of ≥16 mEq. The inclusion criteria included resuscitation for more than 3 min. The infants also had to have clinical signs of encephalopathy within 6 h of birth, in accordance with the National Institute of Child Health and Human Development classification for modified Sarnat staging.[10]

All patients with asphyxia received supportive care under normothermic conditions, which was the standard treatment at the time of recruitment. The exclusion criteria were encephalopathy related aetiologies other than birth asphyxia. These included metabolic diseases and chromosomal abnormalities, as well as confirmed meningitis. The control infants underwent lumber punctures for suspected, but unverified, infections. They all had elevated C-reactive protein in their blood and displayed clinical symptoms that could represent an infection, in conjunction with negative bacterial blood and CSF cultures.

2.2 | Clinical evaluation

Neurological assessments were performed on all patients and controls, according to the Sarnat and Sarnat criteria,[10] before they were enrolled and these were repeated on day 7 of life. The neurological assessment was repeated on the HIE patients at 12, 36 and 72 h of age in the neonatal intensive care unit. All assessments were performed by the same neonatologist.

A neurodevelopmental follow-up was performed by an experienced paediatric neurologist, who examined all the surviving patients at 3, 6 and 18 months of age. The patients who had signs of abnormal neurodevelopment at 18 months of age were assessed with the Bayley Scales of Infant and Toddler Development, Second Edition.[11] Adverse neurological outcomes were defined as: cerebral palsy, a seizure disorder, a mental developmental index of <85 or being deaf or blind at the 18-month assessment. Information was gathered from outpatient paediatric care centres on the outcomes of the control group when they were 18 months of age. Some of the clinical characteristics of a subgroup of the recruited infants have previously been published.[12]

TABLE 1 Clinical characteristics of asphyxiated neonates and non-asphyxiated controls

	n	GA (week)	Birth weight (kg)	Gender (♀:♂)	APGAR5 (score)*	APGAR10 (score)*	Arterial pH*	BE*
Controls	10	40 (38.8 to 41.7)	3.6 (2.8 to 4.6)	5:5	10 (8 to 10)	10 (9 to 10)	7.4 (7.13 to 7.4)	−2.5 (−4 to 0)
HIE, normal outcome	5	41 (40.3 to 41.9)	3.5 (2.8 to 4.2)	2:3	5 (4 to 6)	6.5 (6 to 7)	7.0 (6.9 to 7.1)	−13 (−18 to −8)
HIE, adverse outcome	13	40 (38.6 to 41.6)	3.6 (2.9 to 4.5)	7:6	3 (0 to 6)	4.5 (2 to 6)	6.7 (6.55 to 7.0)	−22 (−30 to −16)

Note: Data are presented as median (IQR). *$p < 0.0001$.

Gestational age, weight, sex and maternal infection didn't differ between groups. As expected, the HIE group had a lower Apgar score and blood gas pH values, compared with non-asphyxiated infants (*$p < 0.001$).

2.3 | CSF analysis

The CSF samples were obtained within the first 3 days of life. The median times and interquartile ranges were 22.5 (15–42) hours after birth for the asphyxiated infants and 26 (13.3–48) hours for the controls. The samples were spun at 3,000 rpm for 10 min and then the supernatants were stored at −80°C until they were analysed.

Antibody suspension bead array technology was used to conduct a comprehensive profiling of the protein expression in the CSF samples taken following perinatal asphyxia. The suspension bead was created from 220 antibodies, which were the affinity reagents, and these targeted the 178 unique proteins, selected from the Human Protein Atlas (Science for Life Laboratory, Stockholm, Sweden)[13] (Table S1). The proteins that were selected had known associations with hypoxic brain injuries and there were previous indications that they had been used as brain injury biomarkers. The selected proteins all provided high tissue enrichment in the central nervous system and were involved in different brain functions.[6,7] The FlexMap3D instrument (Luminex Corp, Texas, USA) was used to analyse cross-linked interacting proteins in the antibody suspension bead. The relative abundance of proteins is reported as the median fluorescent intensities for each sample and bead identity. Further methodological details can be found in Lindblad et al[7] and Appendix S1.

2.4 | Statistics

Clinical and laboratory variables are presented as medians and IQRs. The Mann–Whitney U test was used to compare the independent groups. The results of the protein array analysis have been reported as median fluorescent intensities and IQRs for each sample and antibody. We also used the Mann–Whitney U test to analyse the differences in CSF protein profiles between the patients and controls. No normalisation was performed, due to the low numbers of samples, and this meant that raw median fluorescent intensity data were used. To simplify, we further calculated log2- transformed fold changes of protein levels, visualised as a Volcano plot, to analyse the differences between patients and controls.

We performed principal component analysis to reduce the number of dimensions spanned by the 178 proteins that we measured. The analysis was carried out in R, version 4.0.3 (R Foundation, Vienna, Austria), with the FactoMineR package, version 1.34 (R Foundation).[14] The patients were grouped by their HIE grades and outcomes. The projections of loadings onto the line of best fit were used as a measure of the contribution of each protein to the separation between patients, according to their HIE grade and outcome, respectively.

We compared the groups of patients with adverse outcomes, patients with normal outcomes and controls, using the Kruskal-Wallis test and then used Dunn's multiple comparison test to show differences in the rank sums. The sequentially rejective Bonferroni was used to control for the false discovery rate of multiple testing.[15] The

differences were considered statistically significant if the p value was <0.05. The results are presented on scatter plots that show the differences in protein levels. Pathway analysis was conducted using the R pathfinder package (R Foundation).[16] All proteins were eligible for analysis and the threshold for being included in the input was $p < 0.05$. The enrichment analysis was performed using the BioCarta gene set (BioCarta, Charting pathways of life. http://www.biocarta.com) and results were filtered at $p < 0.05$ after Bonferroni adjustment. The BioGRID (Biological General Repository for Interaction Datasets, thebiogrid.org) protein-protein interaction network was used.

2.5 | Ethics

This study was performed in accordance with European Community guidelines and the Declaration of Helsinki. It was approved by the regional ethics committees at the Karolinska Institute and Stockholm County (Dnr 98–246, 2003–174, 2011/1891-31). Informed, written consent was obtained from the parents of the enrolled patients.

3 | RESULTS

The patient characteristics are summarised in Table 1. This shows that 7 patients had severe HIE (HIE-III), 7 had moderate HIE (HIE-II) and 4 had mild HIE (HIE-I), according to the Sarnat et Sarnat classification of clinical signs.[10] Five patients died during the neonatal period, 8 patients had survived with adverse neurological outcomes by the time of the 18-month follow-up evaluation and 5 patients had normal outcomes. All the non-asphyxiated infants in the control group had normal outcomes.

The relative protein abundance detected in the CSF samples was measured as median fluorescent intensities for each antibody. A distinct CSF proteome, which reflected hypoxic-ischaemic brain injury characteristics, was observed following asphyxia, as several unique proteins were altered in CSF compared with controls (Figure 1A). A differential analysis was performed to compare the outcome groups with regard to the clinical importance of the protein signature in the CSF following perinatal asphyxia. This found that 51 unique CSF proteins correlated with unfavourable outcomes (p value <0.05) (Figure 1B, Table S2). Furthermore, there was an upregulation of neuroinflammatory pathways in CSF following asphyxia, which demonstrated a distinct inflammatory profile compared to the non-asphyxiated controls. Pathway analysis confirmed the importance of immune related proteins (Figure 1C-D). The complement pathway was the most important pathway when it came to discriminating between both HIE grades and outcomes.

A principal component analysis was applied to the data to reduce the number of dimensions spanned by all of the proteins (Figure 2A-B). When the data were grouped by HIE grade and outcomes, both revealed almost identical paths and these created similar gradients from favourable to unfavourable HIE grades and outcomes, respectively. The projections of loadings onto a line of best fit of the centroids are outlined in Table S3. This effectively measured the contribution of each protein to the separation of the data along the favourable to unfavourable gradient. These have been expressed as alpha coefficients. A strong correlation was observed between the principal component analysis alpha coefficients for the proteins that contributed strongly to the differences in data. These were evident in both the fold changes and the p-values on the volcano plot, (Figure 2C-D). Several proteins made a high contribution to the separation between the groups we examined. These included structural proteins, like myelin basic protein and alpha spectrin-II. They also included proteins related to the energy turnover of cells and hypoxic regulation, like neuron-specific enolase, Aldolase C and the ATPase H^+ transporting V1 subunit G2. The list also comprised several synaptic regulating proteins. Table 2 displays the proteins that exhibited the biggest changes in median fluorescent intensities and fold changes between the patients with unfavourable outcomes and control infants (p value <0.005). No differences in median fluorescent intensities were seen between the proteins in patients with normal outcomes and the control infants, apart from beta-synuclein, which was higher in patients than in controls (data not shown). The four proteins that differed most between outcome groups are show in Figure 3A-D.

4 | DISCUSSION

We used an antibody array to analyse 178 proteins related to the central nervous system and inflammation in CSF samples from infants with perinatal asphyxia and non-asphyxiated controls infants. This sensitive measure of the composition CSF proteins identified differences in the concentrations of 51 proteins that correlated with death or adverse neurological outcomes following perinatal asphyxia. The protein profiles that we observed reflected biochemical changes in the CSF, which is in direct contact with the extracellular matrix of the brain, as opposed to blood analyses, which may not reflect events in the central nervous system.[17] Proteins that indicated brain injuries were identified and a clear relationship was determined between the protein concentrations and both the HIE grades and outcomes in patients.

4.1 | Metabolic proteins

We confirmed previous studies that highlighted the importance of proteins that are related to the metabolism of brain cells in hypoxic brain injuries, including neuron-specific enolase, Aldolase C and ATPase H^+ transporting V1 subunit G2. Neuron-specific enolase, which is involved in glycolytic energy metabolism, is an established brain-specific marker of neuronal damage[18] and has been correlated with the risk of death or severe neurological impairment in HIE.[19] It is a commonly used biomarker for traumatic brain injuries[20] and is used in guidelines for managing cerebral anoxia following cardiopulmonary resuscitation in adults, where increasing levels in serum predict an

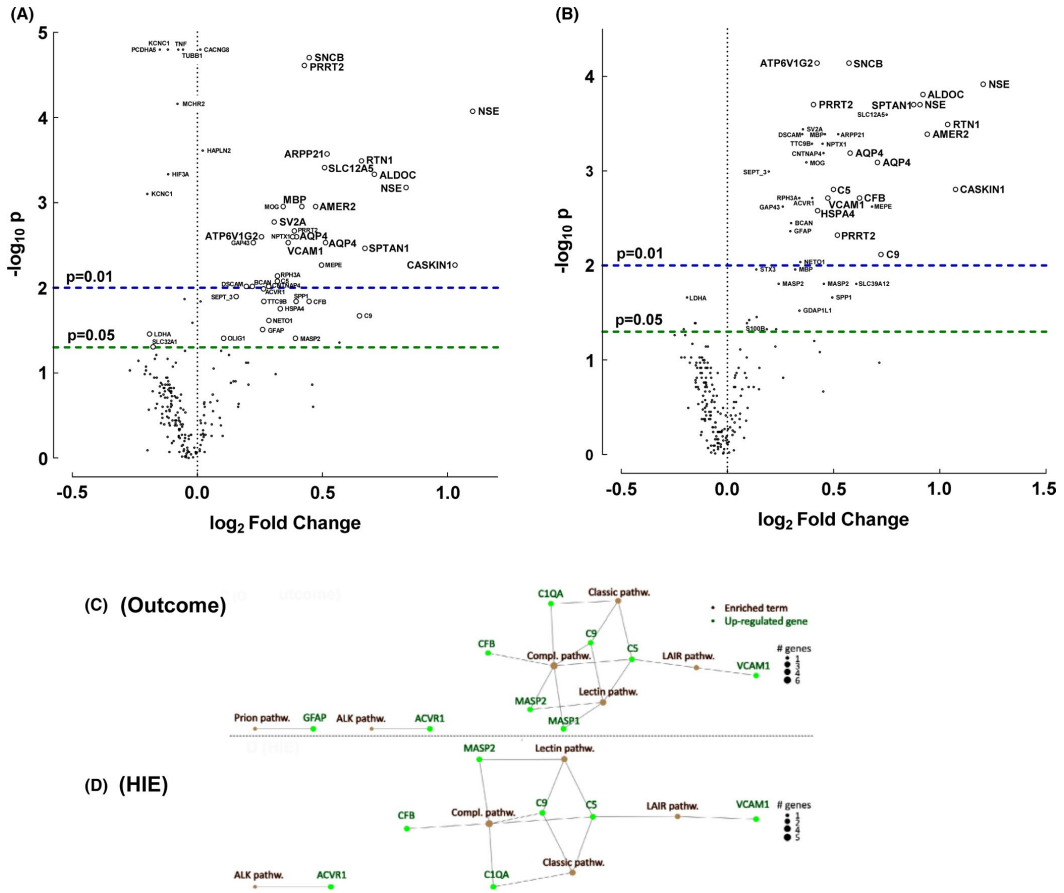

FIGURE 1 Volcano plots describe the relative abundances of CSF proteins in infants with hypoxic-ischaemic encephalopathy (HIE) and controls. The Mann–Whitney U test was used to calculate the differences in median fluorescent intensity (MFI) for each analyte, transform them into $\log_2$ fold changes, which are above zero when increased in HIE, and plot them against $-\log_{10}$ p-values. Figure 1A describes the differences between the 18 HIE patients and 10 controls. Figure 1B describes the differences between HIE patients with adverse outcome and the controls. Pathway analysis shows the upregulation of neuroinflammatory proteins in CSF following perinatal asphyxia, reflected in lectin, complement and classical pathways. The complement pathway was the most important pathway in both outcomes (C) and HIE grades (D)

unfavourable outcome.[21] Secondary ischaemic injuries are common following severe traumatic brain injuries.[22] These are probably due to the deranged cerebral metabolism caused by a regional cerebral mismatch between perfusion and metabolic demand. This is a pathology shared with anoxic injuries and presumably with HIE as well. Aldolase C, a primarily astrocytic protein, is released when there is an astrocyte injury. It has been indicated as a marker of brain damage following traumatic brain injuries and hypoxic ischaemia in animal models.[6,23] A proteomic screening of human adult CSF following traumatic brain injury identified Aldolase C as one of the most promising biomarkers of cell death and functional outcome.[24] This could have a clinical use in HIE. ATPase H^+ transporting V1 subunit G2, which is involved in cell metabolic turnover, has been associated with chronic and progressive traumatic brain injuries with a delayed onset of symptoms.[25] These proteins might indicate the metabolic derangement preceding the secondary phase of a hypoxic-ischaemic brain injury, which leads to mitochondrial impairment and eventually apoptotic neuronal death. This might be of value in clinical decision making, because this time point in the pathological process has been referred to as the window of opportunity for therapeutic interventions.[26] Metabolic derangements during hypoxic ischaemia may lead to disrupted synaptic function, which can induce excitotoxicity and exacerbate brain damage.

4.2 | Synaptic proteins

Several synaptic associated regulatory proteins were increased in our study and correlated with adverse outcomes. None of these

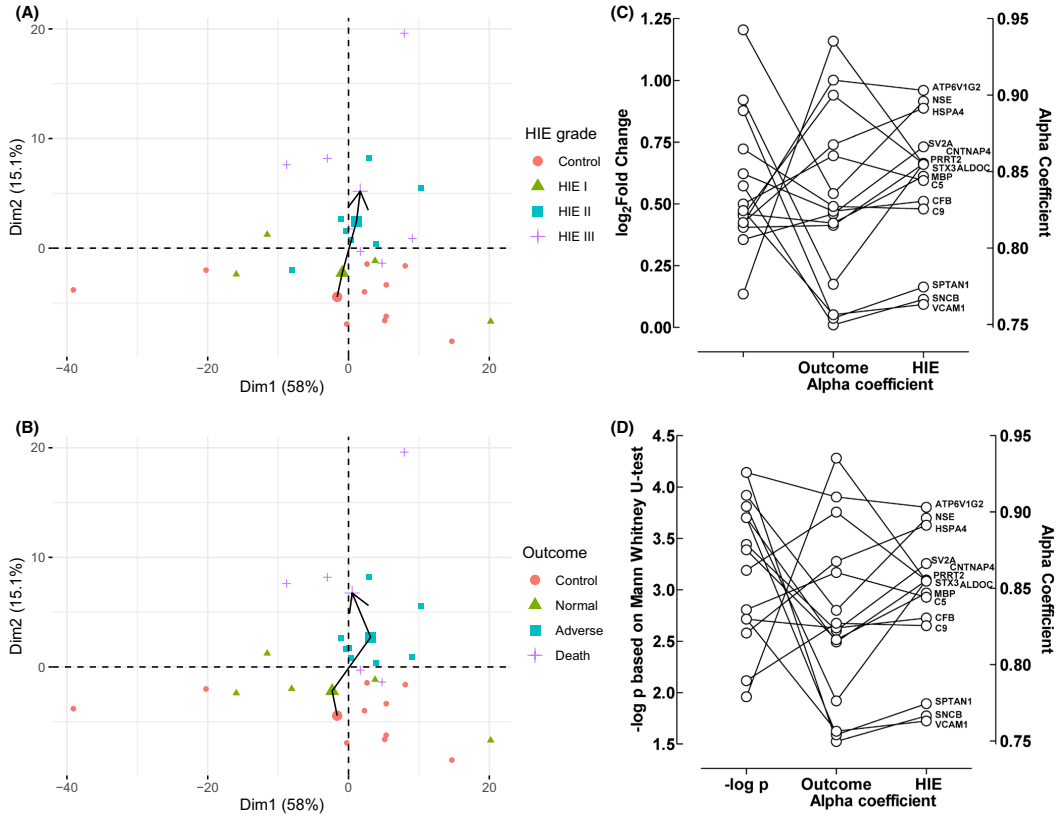

FIGURE 2 The principal component analysis (PCA) score plot of all 178 proteins in the CSF of patients with hypoxic-ischaemic encephalopathy (HIE) and controls. Patients were grouped by either HIE grades (A) or outcomes (B). Centroids are depicted as enlarged symbols and connected in paths from most to least favourable. The contribution of each protein to the separation of the data is expressed as alpha coefficients. Dim1 and Dim2 are the first two principal components. Relationships are described between the alpha coefficients on PCA and fold changes (FC) and expressed on volcano plots (C) and -$\log_{10}$ *p*-values (D). Fold changes describe the $\log_2$ transformed median fluorescent intensity (MFI) differences between HIE patients and controls. Values are based on Mann–Whitney U test

proteins have previously been investigated in relation to HIE. These include synaptic vesicle glycoprotein 2A, a regulator of neurotransmitter release, and reticulon-1, which takes part in excitotoxic neurotransmitter release and may mediate brain damage in hypoxia ischaemia through apoptosis.[27] They also include beta-synuclein, which plays a detrimental role in Alzheimer's disease and Parkinson's disease.[28] Nevertheless, agents that have the potential to reduce excitotoxicity are currently under investigation as promising HIE therapies.[29]

4.3 | Cytoskeletal proteins

Cytoskeletal proteins are released when there is cellular damage or death, and this means that they may serve as markers of brain damage. The myelin basic protein and the alpha II-spectrin protein both increased following perinatal asphyxia and were correlated with unfavourable outcomes. Myelin basic protein is an essential component of the myelin sheath and myelin damage has been correlated with white matter injuries and epilepsy.[30] A correlation has been indicated between increased myelin basic protein levels in serum and CSF and adverse outcomes in paediatric traumatic brain injuries and HIE.[31,32] The same correlation has been seen in traumatic brain injuries and hypoxic-ischaemic brain injury models,.[33,34] Alpha II-spectrin is a protein that is essential for maintaining the integrity of brain cells, as it provides a link between the cytoskeleton and the plasma membrane.[35] It is a novel biomarker for neonatal HIE. It is notable that the present data are in line with suggestions that alpha II-spectrin might be a promising biomarker of brain injuries in infants following cardiac operations[36] and in paediatric traumatic brain injuries.[37] Furthermore, spectrin breakdown products have been shown to exist as exosomes in CSF when adults sustain traumatic

TABLE 2 Protein alterations predicting outcome following perinatal asphyxia

			HIE patients with adverse outcome		Control				
Analyte	Protein description	Function	Median	IQR	Median	IQR	ΔMFI	Log_2 Fold Change	p MWu-test
SNCB	Beta-synuclein	4&5	2553	1420–1783	1717	1420–1783	837	0.5727	7.21E−05
ATP6V1G2	V-type proton ATPase subunit G2	2	1194	1022–1338	891	837–915	303	0.4221	7.21E−05
NSE	Neuron-specific enolase	1&2	2231	1900–3764	968	887–1070	1263	1.2046	1.21E−04
ALDOC	Aldolase C	1&2	1578	1275–1951	834	758–984	745	0.9208	1.55E−04
SPTAN1	Spectrin alpha chain	1&3	760	525–1138	414	355–458	346	0.8772	1.98E−04
PRRT2	Proline-rich transmembrane protein 2	5	772	1240–1757	583	832–1299	189	0.4051	1.98E−04
SLC12A5	Solute carrier family 12 member A5	5	1610	1226–2209	957	783–1061	653	0.7501	2.53E−04
RTN1	Reticulon−1	4&5	1480	963–2357	721	649–768	759	1.0370	3.22E−04
SV2A	Synaptic vesicle glycoprotein 2A	5	1029	909–1298	804	705–899	225	0.3555	3.62E−04
ARPP21	cyclic AMP-regulated phosphoprotein 21	2&5	1147	1057–2248	799	686–919	348	0.9408	4.08E−04
DSCAM	Down syndrome cell adhesion molecule	7&8	582	492–867	456	371–474	126	0.5216	4.08E−04
AMER2	APC membrane recruitment protein 2	7	1003	598–2183	523	476–555	481	0.4594	4.08E−04
MBP	Myelin basic protein	1&3	792	728–1004	576	474–606	216	0.3528	4.08E−04
TTC9B	Tetratricopeptide repeat domain 9B	8	732	610–1078	556	507–600	177	0.4483	5.15E−04
NPTX1	Neuronal pentraxin−1	4	2078	1884–2377	1523	1437–1786	555	0.3981	5.15E−04
AQP4	Aquaporin−4	6&7	5592	4707–7303	3747	2663–4056	1845	0.5775	6.47E−04
CNTNAP4	Contactin 4	5&7	718	618–854	525	409–572	193	0.4520	6.47E−04
MOG	Myelin oligodentrocyte glycoprotein	8	1486	1355–1678	1149	1019–1270	337	0.3714	8.11E−04
CASKIN1	CASK-interacting protein 1	5	2153	1864–3528	1023	845–1960	1131	1.0742	1.56E−03
C5	Complement factor 5	7	970	813–1124	686	603–812	284	0.4993	1.56E−03
CFB	Complement factor B	7	2773	2232–3377	1802	1711–2501	971	0.6220	1.93E−03
VCAM1	Vascular adhesion molecule	7	1609	1260–1733	1159	768–1447	450	0.4730	1.93E−03
ACVR1	Activin A receptor type 1	8	1152	1003–1322	873	802–1033	279	0.3995	1.93E−03
RPH3A	Rabphilin 3A	5	693	626–919	548	475–637	145	0.3387	1.93E−03
GAP43	Growth associated protein 43	8	7728	7277–9030	6451	4534–6745	1277	0.2606	2.37E−03
MEPE	Matrix extracellular phophoglycoprotein	3	1074	791–1777	670	548–876	404	0.6808	2.37E−03
HSPA4	Heat shock protein family A member 4	7	551	473–756	411	328–428	141	0.4247	2.63E−03

Note: Proteins in CSF that exhibited statistically significant differences in median fluorescent intensity (MFI) levels between patients with adverse outcome and controls at threshold of $p < 0.005$, established by Mann–Whitney (MW) u-test and the sequentially rejective Bonferroni test ($\alpha = 0.005$). 1 = Marker of brain cellular damage; 2 = Energy metabolism; 3 = Brain cell structure; 4 = Apoptotic properties; 5 = Synaptic regulation; 6 = Brain vascular regulation; 7 = Neuroinflammation; 8 = Neurotrophic properties.

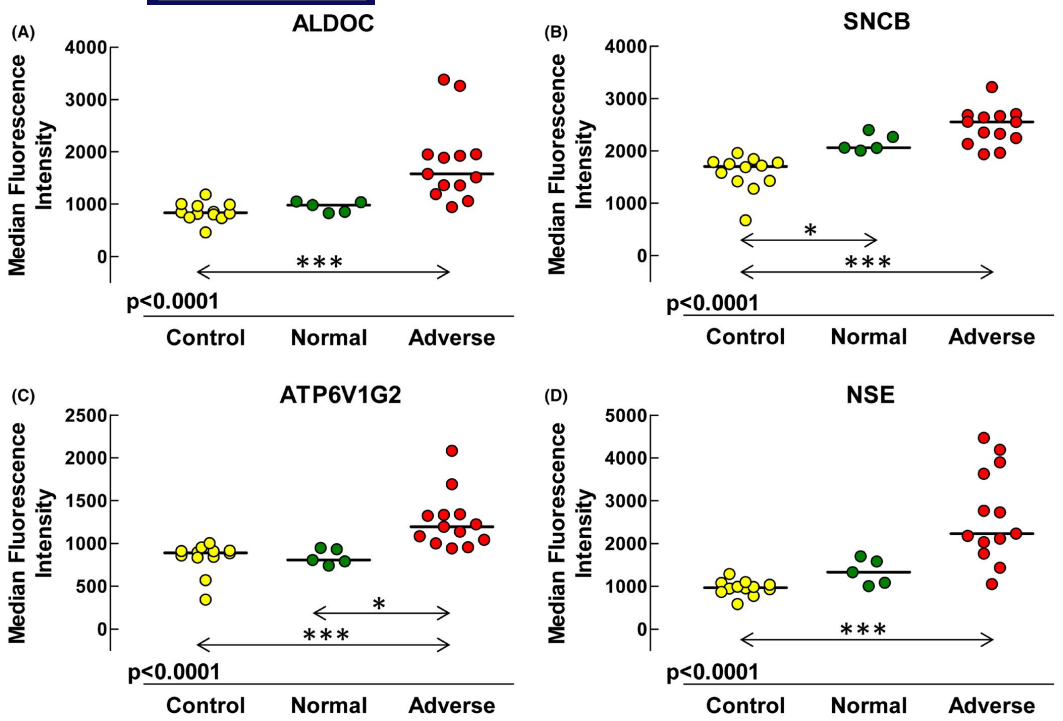

FIGURE 3 Scatter plot of the proteins that exhibited the largest differences between the outcome groups: aldolase C (ALDOC) (A), Beta-synuclein (SNCB) (B), ATPase H^+ transporting V1 subunit G2 (ATP6V1G2) (C) and neuron-specific enolase (NSE) (D). Adverse outcomes are defined as death or abnormal neurological outcomes at 18 months. Data were analysed with the Kruskal-Wallis test and Dunn's Multiple Comparison Test ($p < 0.0001$)

brain injuries. They function as cell death signalling molecules in the pathological pathways, leading to neurodegeneration.[38]

4.4 | Neuroinflammatory pathway proteins

The key cellular pathways of hypoxic-ischaemic brain injuries include the upregulation of the innate immune system. Inflammatory mediators may be produced within minutes of a brain insult and continue to expand for weeks and even months. They are the main contributors to the chronic prolonged phase of the injury, when the regeneration and repair of neurons may be prevented and neurodevelopment altered.[4] Clinical and experimental data that underline the importance of inflammatory mediators in perinatal brain injuries continue to emerge.[12,39,40] The present study found that increased levels of several inflammatory biomarkers correlated with unfavourable outcomes in patients. Furthermore, pathway analysis confirmed the importance of the complement pathway. It is of upmost importance to recognise the neuroinflammatory reaction in hypoxic-ischaemic brain injuries, as this may open up new possibilities for therapeutic interventions.

4.5 | Strengths and limitations

The study´s main strength was that we used a protein array, which is an emerging technique in hypoxic-ischaemic brain injuries. Doing this enabled us to provide novel insights into the underlying pathophysiology of brain disease. This technique also enabled us to simultaneously quantify 178 proteins in small CSF samples.

The study also had several limitations that must be acknowledged. It is important to point out that there was a time lapse between recruiting the patients and analysing the samples, as well as presenting the results. This means that it is possible that some of the frozen protein samples deteriorated over time. Also, hypothermia was not a standard treatment for HIE at the time of recruitment, so we did not have cooled infants in our patient group. On the other hand, this could have provided us with important information about brain pathology without the influence of therapeutic hypothermia. Another limitation was that the developmental evaluation, carried out with the Bayley Scales of Infant and Toddler Development, Second Edition, was only performed on infants with abnormal neurological symptoms at 18 months of age. Infants without symptoms did not undergo this test.

5 | CONCLUSION

This study has demonstrated an unprecedented array of CSF proteomic profiling alterations in protein levels following perinatal asphyxia and showed that these were associated with the severity of HIE and long-term outcomes. These can provide biomarkers for perinatal asphyxia. Several of these proteins are novel biomarkers for long-term outcomes after HIE and will require external validation in larger patient cohorts. Alterations in several novel biomarkers have previously been observed in biofluids in similar cerebral conditions, like traumatic brain injuries. This suggests a shared pathophysiology. Our study also characterised the pathological pathways involved in perinatal asphyxia, and this may open up new therapeutic options for reducing long-term morbidity and mortality.

As a result of our findings, we suggest that these markers should be used to monitor different pathophysiological processes following HIE. This could present tentative treatment options, but further research is warranted.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request in a format that adheres to current Swedish and European Union legislation regarding study participant anonymity.

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REFERENCES

1. Liu L, Oza S, Hogan D, et al. Global, regional, and national causes of under-5 mortality in 2000–15: an updated systematic analysis with implications for the Sustainable Development Goals. Lancet. 2016;388(10063):3027-3035.
2. Lundgren C, Brudin L, Wanby AS, Blomberg M. Ante- and intrapartum risk factors for neonatal hypoxic ischemic encephalopathy. J Matern Fetal Neonatal Med. 2018;31(12):1595-1601.
3. Wassink G, Davidson JO, Lear CA, et al. A working model for hypothermic neuroprotection. J Physiol. 2018;596(23):5641-5654.
4. Hagberg H, Mallard C, Ferriero DM, et al. The role of inflammation in perinatal brain injury. Nat Rev Neurol. 2015;11(4):192-208.
5. Satriano A, Pluchinotta F, Gazzolo F, Serpero L, Gazzolo D. The potentials and limitations of neuro-biomarkers as predictors of outcome in neonates with birth asphyxia. Early Hum Dev. 2017;105:63-67.
6. Thelin EP, Just D, Frostell A, et al. Protein profiling in serum after traumatic brain injury in rats reveals potential injury markers. Behav Brain Res. 2018;340:71-80.
7. Lindblad C, Pin E, Just D, et al. Fluid proteomics of CSF and serum reveal important neuroinflammatory proteins in blood-brain barrier disruption and outcome prediction following severe traumatic brain injury: a prospective, observational study. Crit Care. 2021;25(1):103.
8. Boardman JP, Ireland G, Sullivan G, et al. The Cerebrospinal Fluid Inflammatory Response to Preterm Birth. Front Physiol. 2018;9:1299.
9. Uhlen M, Oksvold P, Fagerberg L, et al. Towards a knowledge-based Human Protein Atlas. Nat Biotechnol. 2010;28(12):1248-1250.
10. Sarnat HB, Sarnat MS. Neonatal encephalopathy following fetal distress. A clinical and electroencephalographic study. Arch Neurol. 1976;33(10):696-705.
11. Cirelli I, Bickle Graz M, Tolsa JF. Comparison of Griffiths-II and Bayley-II tests for the developmental assessment of high-risk infants. Infant Behav Dev. 2015;41:17-25.
12. Leifsdottir K, Mehmet H, Eksborg S, Herlenius E. Fas-ligand and interleukin-6 in the cerebrospinal fluid are early predictors of hypoxic-ischemic encephalopathy and long-term outcomes after birth asphyxia in term infants. J Neuroinflammation. 2018;15(1):223.
13. Collaborators. Human protein atlas. https://www.proteinatlas.org/ Published 2021, November 18 Accessed
14. R Core Team. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. https://www.r-project.org/ Published 2021 Accessed
15. Benjamini Y, Drai D, Elmer G, Kafkafi N, Golani I. Controlling the false discovery rate in behavior genetics research. Behav Brain Res. 2001;125(1-2):279-284.
16. Ulgen E, Ozisik O, Sezerman OU. pathfindR: An R Package for Comprehensive Identification of Enriched Pathways in Omics Data Through Active Subnetworks. Front Genet. 2019;10:858.
17. Ellison VJ, Mocatta TJ, Winterbourn CC, Darlow BA, Volpe JJ, Inder TE. The relationship of CSF and plasma cytokine levels to cerebral white matter injury in the premature newborn. Pediatr Res. 2005;57(2):282-286.
18. Shaik AJ, Reddy K, Mohammed N, Tandra SR, Rukmini Mridula K, Baba KS. Neuron specific enolase as a marker of seizure related neuronal injury. Neurochem Int. 2019;131:104509.
19. Kelen D, Andorka C, Szabo M, Alafuzoff A, Kaila K, Summanen M. Serum copeptin and neuron specific enolase are markers of neonatal distress and long-term neurodevelopmental outcome. PLoS One. 2017;12(9):e0184593.
20. Thelin EP, Jeppsson E, Frostell A, et al. Utility of neuron-specific enolase in traumatic brain injury; relations to S100B levels, outcome, and extracranial injury severity. Crit Care. 2016;20:285.
21. Wijdicks EF, Hijdra A, Young GB, Bassetti CL, Wiebe S. Quality Standards Subcommittee of the American Academy of N. Practice parameter: Prediction of outcome in comatose survivors after cardiopulmonary resuscitation (an evidence-based review): Report of the Quality Standards Subcommittee of the American Academy of Neurology. Neurology. 2006;67(2):203-210.
22. Thelin EP, Nelson DW, Bellander BM. Secondary peaks of S100B in serum relate to subsequent radiological pathology in traumatic brain injury. Neurocrit Care. 2014;20(2):217-229.
23. Ottens AK, Bustamante L, Golden EC, et al. Neuroproteomics: a biochemical means to discriminate the extent and modality of brain injury. J Neurotrauma. 2010;27(10):1837-1852.
24. Halford J, Shen S, Itamura K, et al. New astroglial injury-defined biomarkers for neurotrauma assessment. Journal of Cerebral Blood

Flow and Metabolism: Official Journal of the International Society of Cerebral Blood Flow and Metabolism. 2017;37(10):3278-3299.
25. Harper MM, Rudd D, Meyer KJ, et al. Identification of chronic brain protein changes and protein targets of serum auto-antibodies after blast-mediated traumatic brain injury. Heliyon. 2020;6(2):e03374.
26. Gunn AJ, Bennet L. Brain cooling for preterm infants. Clin Perinatol. 2008;35(4):735–748.
27. Gong L, Tang Y, An R, Lin M, Chen L, Du J. RTN1-C mediates cerebral ischemia/ reperfusion injury via ER stress and mitochondria-associated apoptosis pathways. Cell Death Dis. 2017;8(10):e3080.
28. Sargent D, Betemps D, Drouyer M, et al. Investigating the neuroprotective effect of AAV-mediated beta-synuclein overexpression in a transgenic model of synucleinopathy. Sci Rep. 2018;8(1):17563.
29. Filippi L, Fiorini P, Catarzi S, et al. Safety and efficacy of topiramate in neonates with hypoxic ischemic encephalopathy treated with hypothermia (NeoNATI): a feasibility study. J Matern Fetal Neonatal Med. 2018;31(8):973-980.
30. You Y, Bai H, Wang C, et al. Myelin damage of hippocampus and cerebral cortex in rat pentylenetetrazol model. Brain Res. 2011;1381:208-216.
31. Beers SR, Berger RP, Adelson PD. Neurocognitive outcome and serum biomarkers in inflicted versus non-inflicted traumatic brain injury in young children. J Neurotrauma. 2007;24(1):97-105.
32. Garcia-Alix A, Cabanas F, Pellicer A, Hernanz A, Stiris TA, Quero J. Neuron-specific enolase and myelin basic protein: relationship of cerebrospinal fluid concentrations to the neurologic condition of asphyxiated full-term infants. Pediatrics. 1994;93(2):234-240.
33. Chen Y, Yi Q, Liu G, Shen X, Xuan L, Tian Y. Cerebral white matter injury and damage to myelin sheath following whole-brain ischemia. Brain Res. 2013;1495:11-17.
34. Ottens AK, Golden EC, Bustamante L, Hayes RL, Denslow ND, Wang KK. Proteolysis of multiple myelin basic protein isoforms after neurotrauma: characterization by mass spectrometry. J Neurochem. 2008;104(5):1404-1414.
35. Lubbers ER, Murphy NP, Musa H, et al. Defining new mechanistic roles for alphaII spectrin in cardiac function. J Biol Chem. 2019;294(24):9576-9591.
36. Jain P, Spaeder MC, Donofrio MT, Sinha P, Jonas RA, Levy RJ. Detection of alpha II-spectrin breakdown products in the serum of neonates with congenital heart disease*. Pediatr Crit Care Med. 2014;15(3):229-235.
37. Berger RP, Hayes RL, Richichi R, Beers SR, Wang KK. Serum concentrations of ubiquitin C-terminal hydrolase-L1 and alphaII-spectrin breakdown product 145 kDa correlate with outcome after pediatric TBI. J Neurotrauma. 2012;29(1):162-167.
38. Manek R, Moghieb A, Yang Z, et al. Protein Biomarkers and Neuroproteomics Characterization of Microvesicles/Exosomes from Human Cerebrospinal Fluid Following Traumatic Brain Injury. Mol Neurobiol. 2018;55(7):6112-6128.
39. Li B, Concepcion K, Meng X, Zhang L. Brain-immune interactions in perinatal hypoxic-ischemic brain injury. Prog Neurogibol. 2017;159:50-68.
40. Bjork L, Leifsdottir K, Saha S, Herlenius E. PGE-metabolite levels in CSF correlate to HIE score and outcome after perinatal asphyxia. Acta Paediatr. 2013;102(11):1041-1047.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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Paper V

The cerebrospinal fluid proteome of preterm infants predicts neurodevelopmental outcome

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ABSTRACT

Background: Survival rate increases for preterm infants, but long-term neurodevelopmental outcome predictors are lacking. Our primary aim was to determine whether a specific proteomic profile in cerebrospinal fluid (CSF) of preterm infants differs from that of term infants and to identify novel biomarkers of neurodevelopmental outcome in preterm infants.
Methods: Twenty-seven preterm infants with median gestational age 27w+4d and ten full-term infants were enrolled prospectively. Protein profiling of CSF were performed utilizing an antibody suspension bead array. The relative levels of 178 unique brain derived proteins and inflammatory mediators, selected from the Human Protein Atlas, were measured.
Results: The CSF protein profile of preterm infants differed from that of term infants. Increased levels of brain specific proteins that are associated with neurodevelopment and neuroinflammatory pathways made up a distinct protein profile in the preterm infants. The most significant differences were seen in proteins involved in neurodevelopmental regulation and synaptic plasticity, as well as components of the innate immune system. Several proteins corralated with favorable outcome in preterm infants at 18-24 months corrected age. Among the proteins that provided strong predictors of outcome were vascular endothelial growth factor C, Neurocan core protein and seizure protein 6, all highly important in normal brain development.
Conclusions: Our data suggest a vulnerability of the preterm brain to postnatal events and that alterations in protein levels may contribute to unfavorable neurodevelopmental outcome.

INTRODUCTION

Despite improvement in the care of preterm infants during the last decades, neurodevelopmental deficiencies remain a major cause of chronic neurological morbidity throughout life (Bell et al., 2022; Cheong et al., 2021). The degree of immaturity at birth and continued brain development in the neonatal period, determines long-term neurological outcome of preterm infants (Chau et al., 2013). Insults during the neonatal period may alter these developmental processes. However, the diagnosis of preterm brain injury and outcome prediction are difficult to perform. Brain abnormalities diagnosed with neuroimaging techniques correlate with adverse outcome, but normal diagnostic findings do not necessarily predict normal neurodevelopment (Mathur & Inder, 2009). Infants born prematurely have decreased brain volume at term-equivalent age on magnetic resonance imaging (MRI) compared to term infants (Thompson et al., 2019). This might indicate a suboptimal neurodevelopment after preterm birth. Furthermore, alterations in microstructural neural connectivity, not captured on imaging-defined volume measures, are important promoters of disturbed brain development (Chau et al., 2012; Gozdas et al., 2018). Microstructural white matter disorganization has even been observed in adolescents who were born preterm, correlating with lower scores of language function compared with former term controls (Mullen et al., 2011).

Not only functional and structural brain enriched proteins, but also many immune proteins are present in the brain. They have a role in brain development and synaptic plasticity (Boulanger, 2009). While inflammatory cascades may become detrimental, inflammatory proteins are part of the host defense system, with beneficial functions. It has also been suggested that inflammation may mediate neuroprotection by preconditioning the developing brain (Mallard & Hagberg, 2007). Increased levels of inflammatory proteins in CSF has been correlated with preterm birth (Boardman et al., 2018).

Recently, novel techniques for sensitive protein analysis in body fluids, like antibody-based proteomic targeting, have emerged. These techniques are increasingly utilized for comprehensive examination of CSF, with the purpose of discovering biomarkers of brain pathology (Pin et al., 2019). Changes in the highly active central nervous system (CNS) are reflected in biochemical alterations in CSF, that is in direct contact with the extracellular matrix of the brain (Ellison et al., 2005). The CSF system is important for the homeostasis of the CNS. It facilitates communication between the central nervous, the vascular, and immune systems. Soluble proteins and other macroscopic metabolic waste products are in part cleared from CSF by a recently discovered glymphatic system (Jessen, Munk, Lundgaard, & Nedergaard, 2015). An affected glymphatic system has been associated with cognitive decline in the elderly (Kress et al., 2014) and there are indications of a causal link between the progression of neurodegenerative diseases and diminished solute clearance (Rasmussen, Mestre, & Nedergaard, 2018).

The two aims of the present study were to evaluate if a targeted profiling of the CSF proteome, primarily including inflammatory and structural brain antigens, would a) differ between preterm and term infants, and b) predict neurodevelopmental outcome in preterm infants at 18 to 24 months corrected age. Our hypothesis was that protein concentrations would be different between the infants born preterm and the infants born at term age. Furthermore, that preterm infants with an unfavorable outcome might present a specific proteomic signature compared with the infants with favorable outcome. This was investigated through quantifications of CSF inflammatory mediators as well as structural and functional proteins related to brain development.

MATERIALS and METHODS

Study participants and data acquisition

Twenty-seven preterm infants and 10 term-born infants were prospectively enrolled from the neonatal intensive care unit at Karolinska Hospital in Stockholm, Sweden, between January 2002 and May 2004, with informed parental consent. The median (inter quartile range (IQR)) gestational age was 27w+4d (25+2 to 30+6) and 40w+6d (38+0 to 41+6) for the preterm and term infants, respectively. All enrolled infants underwent clinically indicated lumbar puncture for suspected infection, and all received antibiotic treatment. Measurement of clinically established markers of infection in blood was performed in correlation with the lumbar puncture. Bacterial and viral cultures were performed in blood and CSF as per clinical routine. Twelve out of 49 eligible infants were excluded for one of the following reasons: intraventricular hemorrhage grade 2 or more according to Papile et al (Papile, Burstein, Burstein, & Koffler, 1978), signs of periventricular leukomalacia on cranial ultrasound around the time of CSF collection, perinatal asphyxia or any other findings that indicated brain encephalopathy.

A neurodevelopmental follow-up was carried out in accordance with the Karolinska neonatal follow up program for all preterm infants. It was performed by a senior neonatologist and a physiotherapist in the time period between 18 and 24 months corrected postnatal age. Infants who presented abnormal neurodevelopment at that time were assessed with Bayley Scales of Infant and Toddler Development, Second Edition (BSID-II) (Bayley, 1993).

All the term infants were examined by a senior neonatologist before discharge. They were not followed further in the neonatal follow-up program but had their check-ups within the regular childcare center, from where the information of the 18-24 month follow up was retrieved.

Adverse neurodevelopmental outcome was defined as: neuromotor delay, cerebral palsy, seizure disorder, mental developmental index < 85, deafness or blindness, for preterm and term infants.

Collection of cerebrospinal fluid (CSF)

The median (IQR) age at CSF collection in preterm infants was 11 postnatal days (3.5 to 19.5d) while at median 2 postnatal days (1 to 2.5d) in term infants. Lumbar punctures were performed for clinical routine laboratory analysis in all patients and an additional 0.2 to 0.8 ml collected for research purposes. Samples were spun at 3,000 revolutions per minute (rpm) for 10 min and then the supernatants stored at −80 °C until analyzed. Before analysis, the samples were thawed, and processed as previously described (Pin et al., 2019).

Cerebrospinal fluid analysis

For the protein profiling in CSF a targeted antibody suspension bead array was used. It included 220 antibodies targeting 178 unique proteins (**Supplementary Table 1**). For the analyzis, highly to moderately brain enriched proteins that are involved in different brain functions as well as a selection of proteins involved in neuroinflammatory processes, as recently described by Lindblad et al (Lindblad et al., 2021) were chosen from the Human Protein Atlas (Science for Life Laboratory, Stockholm, Sweden) (Collaborators, 2021, November 18). Two “sibling”-antibodies targeting different regions of the same protein were included for 43 of the proteins. Antibodies for the creation of the suspension bead array were produced from protein fragments (PrESTs) produced in E.Coli and then immunized into rabbits after purification (23). Activation of the color-coded magnetic beads (500 000 beads per identity, MagPlex Luminex Corp.) and immobilization of the antibodies was performed as previously described (Schwenk, Gry, Rimini, Uhlen, & Nilsson, 2008). Sodium hydrogen phosphate 0.1M, sulfo-NHS (Nordic Biolabs) 0.5mg and EDC (ProteoChem) 0.5mg per antibody were used to activate the beads surface. Then an incubation of the beads for 20 minutes at room temperature was performed in

order to immobilize the selected antibodies to the different bead identities. A total concentration of 17.5 ng/microL of each antibody was assigned a specific bead ID. This was followed by washing off unwanted antibody excess with 0.1% Tween-20 after which the beads were blocked over night using Roche blocking reagent for ELISA (supplemented with 400 microL Tween20) and lastly combined to form a suspension array.
The samples were processed as previously described (Pin et al., 2019). An assay buffer (BSA (Sigma-Aldreick)) 37.5 mg/mL, PBS (Fisher Scientific) 0.05% and rIgG 15 mg/mL (Bathyl Laboratories Inc., Montgomery, Texas, USA) were used to dilute the samples 1:2 after which they were randomized into 96-well microtiter plates. A labelling of the samples was performed using 10x molar excess of biotin (NHS-PEG4-Biotin, Thermo Scientific) over protein amount, as previously described (Haggmark et al., 2013). Tris-HCl, 250x over biotin amount was used to stop the reaction and an assay buffer (PVXCas) used for another 1:8 dilution. The samples were then put in a water bath for 30 minutes heat treatment at 56 °C and after cooling incubated with the bed array overnight at room temperature in combinations of 45 microL of sample with 5 microL of bead array solution. Using paraformaldehyde (0.4% in PBS) the captured proteins were cross-linked to the antibodies. After a second wash a detection reagent (streptavidin conjugated R-phycoerythrin, 1:750 diluted in PBST, Invitrogen) was added. Finally, FlexMap3D instrument (Luminex Corp., Austin, Texas) was utilized to analyze the interacting proteins and report the relative protein abundances for each bead identity and sample as median fluorescent intensities.

Statistics

Median and IQR were used to demonstrate the clinical and laboratory data. The results of the protein array analysis were presented as raw median fluorescent intensity. No normalization was performed, due to the low numbers of samples available. For simplification, the median fluorescent intensity differences of CSF protein abundances were visualized on a Volcano plot after log-2 transformation of the data into fold changes. The differences of the CSF protein levels between preterm and term infants as well as the preterm outcome groups, were analyzed using Mann-Whitney U test. The differences were considered statistically significant if p-value was <0.05. The results of proteins representing the largest median fluorescent intensity differences between preterm and term infants and between preterm outcome groups are presented on scatter plots. $p<0.001$, to be included in figures.

RESULTS

Patient characteristics

The patient characteristics are summarized in **Table 1**. Gestational age, birth weight, postnatal age at lumbar puncture and number of verified infections differed between the preterm and term infant groups but not the C-reactive protein levels. Eleven of the preterm infants had adverse neurological outcome at 18-24 months follow-up assessment, but none of the term infants. While complete outcome data was missing for n=3 preterm infants, national records showed that they were all alive at 24 months. No significant differences were found in gestational age, postnatal age at lumbar puncture or C-reactive protein level in the preterm group divided by outcome (**Table 2**). Sixteen out of 26 preterm infants with available culture results had culture verified blood stream and/or CSF infection, 8/13 (62%) with normal outcomes and 8/11 (72%) with unfavorable outcomes, respectively. All term infants had negative CSF- and blood cultures.

Table 1

Clinical characteristics of preterm and term infants

	Term infants	Preterm infants
Number of patients (n)	10	27
Gestational age (weeks+days)	40+6 (38+0 to 41+5)	27+5 (25+3 to 30+6)
Birth weight (g)*	3900 (3627 to 4943)	850 (707 to 1117)
Age at lumbar puncture (days)*	2 (1 to 2,25)	11 (4 to 21)
C-reactive protein	92 (71 to 110)	75 (14 to 96)
Positive blood or CSF cultures (n)*	0/10	16/26**

Data are presented as median (IQR).
*p < 0.001, ** *n*=26 of the preterm infants had available data on blood and CSF cultures
CSF; cerebrospinal fluid

Table 2

Neurodevelopmental outcome

	Preterm infants with adverse outcome* (*n*=11)	Preterm infants with normal outcome (*n*=13)
Gestational age (weeks+days)	26+3 (25+1 to 28+2)	27+4 (25+2 to 31+0)
Birth weight (g)	912 (707 to 1005)	834 (659 to 1454)
Age at lumbar puncture (days)	8 (2 to 15)	11 (5 to 28)
C-reactive protein	70 (7 to 90,5)	94 (30 to 124)
Positive blood or CSF cultures (n)	8	8

Data are presented as median (IQR).
Adverse outcome was defined as: cerebral palsy, seizure disorder, motor delay, mental developmental index < 85, deafness or blindness at 18 to 24 months assessment.

Differences in protein profiles between preterm and term infants

A distinct CSF proteome reflecting ongoing neurodevelopmental and neuroinflammatory processes was observed in preterm infants, **Figure 1**. The relative protein abundances detected in CSF samples, represented as median fluorescent intensity differences are transformed into $\log_2$ fold changes for visualization.

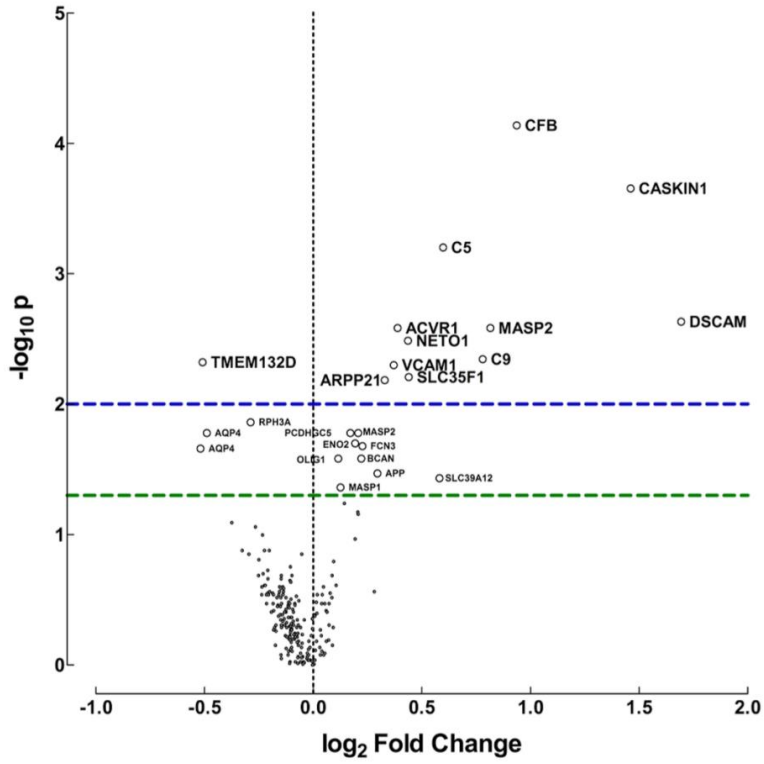

Figure 1. Protein differences between preterm and term infants

Volcano plot for visualization of the differences in relative protein abundances between preterm and term infants. The plot was created by transforming the median fluorescent intensity differences into $\log_2$ fold changes and plot it against $-\log_{10}$ *p*-values, calculated with Mann-Whitney U test. Fold changes above zero indicate higher protein levels in CSF of preterm infants, but below zero indicates higher levels in term infants. Horizontal dashed lines indicate significance, green: p=0.05, blue: p=0.01.

Proteins related to the innate immune response and neurodevelopment exhibited the biggest difference in median fluorescent intensities and fold changes between preterm and term infants. These included complement factor B (CFB), complement 5 (C5), complement 9 (C9) and mannose-binding protein-associated serine protease 2 (MASP). Moreover, proteins involved in neurodevelopmental regulation and synaptic plasticity were also present in higher concentrations in preterm versus term infants. These included calcium/calmodulin-dependent serine protein kinase (CASK)-interacting protein 1 (CASKIN1), cyclic AMP-regulated phosphoprotein 21 (ARPP21), Neurophilin and tolloid like proten 1 (NETO1) and Down syndrome cell adhesion molecule (DSCAM). Only a handful of proteins were found increased in term infants compared with preterm.

The protein differences between preterm and term infants were consistent regardless of culture results. When protein levels were compared in preterm infants with positive and negative cultures, no differences were found (**Supplementary Table 2**). As the term infants all had favorable outcomes, an additional comparison was made between the protein levels of term and preterm infants with normal outcomes. Similar protein concentrations were found as described above, but for most proteins the differences were larger in this subset. Significant differences were found for 35 proteins, p-value <0.05. The proteins that exhibited the highest median fluorescent intensity data differences and lowest p-values, are displayed in **Table 3**. All protein differences are shown in **Supplementary Table 3.**

Table 3. Protein alterations between term and preterm infants with normal outcome

			Preterm		Term				
Analyte	**Description**	**Function**	**Median**	**IQR**	**Median**	**IQR**	**ΔMFI**	**log2 Fold Change**	***p****
CFB	Complement factor B	Neuroinflammation, regulation of immune reaction	3448	3222-4678	1802	1643-2209	**1646**	0.94	7.21E-05
C5	Complement 5	Neurodevelopment and Neuroinflammation	1143	961-1249	686	588-751	**438**	0.74	1.98E-04
CASKIN1	CASK-interacting protein 1	Synaptic plasticity	3753	2294-5863	1022	790-1521	**2731**	1.88	1.98E-04
VCAM1	Vascular cell adhesion molecule 1	Neuroinflammation and early neural proliferation	1672	1478-2002	1159	1105-1411	**513**	0.53	5.15E-04
ARPP21	Cyclic AMP-regulated phosphoprotein 21	Synaptic plasticity	1109	970-1203	799	701-905	**310**	0.47	7.25E-04
MASP2	Mannose-binding protein-associated serine protease 2	Neural migration and Neuroinflammation	1730	1353-3899	982	824-1168	**748**	0.82	8.11E-04
ACVR1	Activin receptor type-1	Neurotrophic & bone morphogenic properties	1165	1075-1431	873	770-953	**292**	0.42	1.26E-03
BCAN	Brevican	Brain developmental & plasticity, maintenance of neural circuitry	1863	1581-1977	1424	1164-1636	**439**	0.39	1.93E-03
NETO1	Neurophilin and tolloid like proten 1	Synaptic plasticity	993	829-1477	712	658-865	**281**	0.48	1.93E-03
DSCAM	Down syndrome cell adhesion molecule	Brain development and Neurotrophic properties	8685	4537-11743	2158	938-4267	**6527**	2.01	1.93E-03
NSE	Neuron specific enolase	Neurotrophic & Neuroprotionproperties	1222	1035-1502	968	892-1085	**254**	0.34	2.37E-03
APP	Amyloid beta precursor protein	Synaptogenesis & Synaptic plasticity	4460	3899-4970	3264	2834-3942	**1196**	0.45	4.33E-03

Proteins in cerebrospinal fluid that exhibited differences in median fluorescent intensity levels at the threshold of $p<0.005$ between term infants and preterm infants with outcome, established by Mann- Whitney U test. Abbreviations; ΔMFI = median fluorescent intensity differences, IQR = inter quartile range. *p = <0.005.

The four inflammatory proteins that differed the most between these patient groups are displayed in more detail in **Figure 2**.

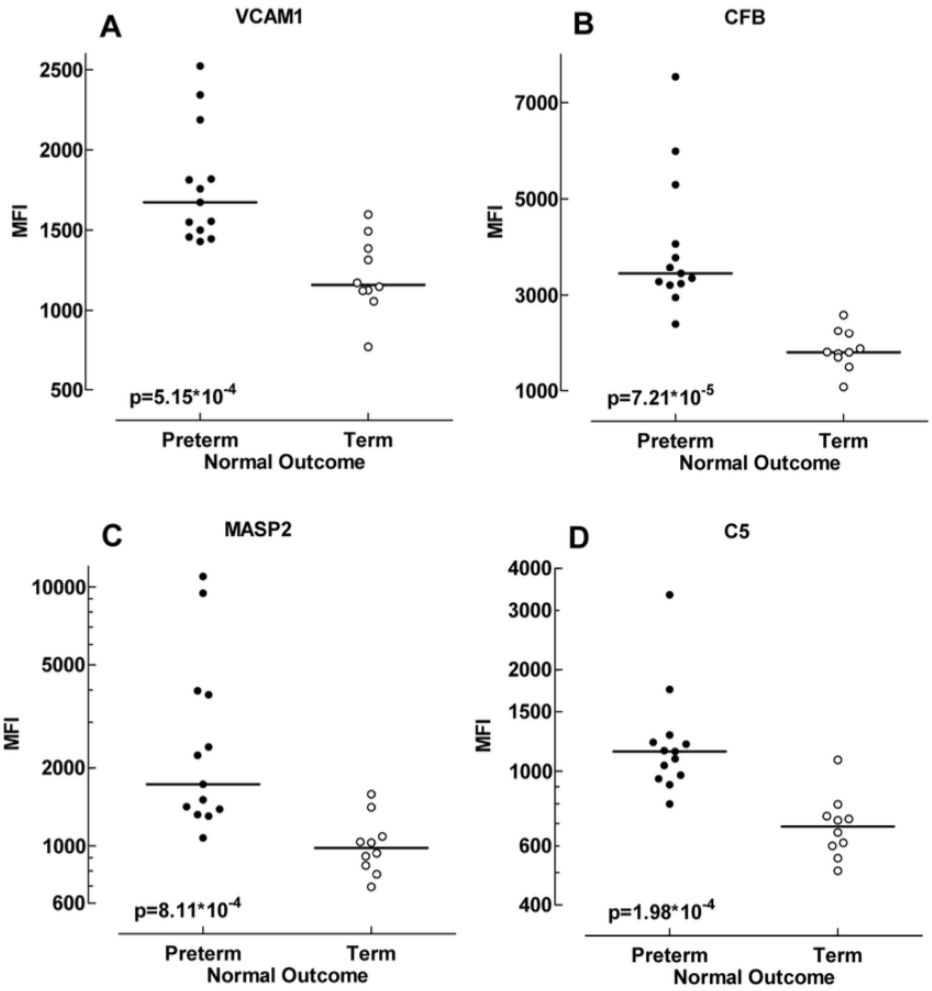

Figure 2. Protein differences between term and preterm infants with normal outcome
Scatter plot visualizing alterations in protein levels between preterm and term infant groups. Four of the proteins exhibiting the largest data differences; VCAM1 (vascular cell adhesion molecule 1) (**A**), CFB (complement factor B) (**B**), MASP2 (mannose-binding protein associated serine protease 2) (**C**) and C5 (complement factor 5) (**D**). Data analyzed with Mann-Whitney U test, $p<0.001$.

Proteins predicting outcome in preterm infants

A differential analysis was conducted to compare the protein levels in pretem infants with favorable and unfavorable outcomes. Several proteins were negatively correlated with unfavorable outcome in preterm infants (**Supplementry Table 4**). In **Table 4** the proteins that differed the most between the outcome groups are highlighted ($p<0.001$). Most are proteins essential for brain function and development. These proteins were consistently found at lower

levels in preterm infants with adverse outcome, regardless of culture results (data not shown). In **Figure 3**, four of the proteins with the biggest alterations in median fluorescent intensity between the outcome groups are displayed.

Table 4. Protein alterations predicting outcome in preterm infants

			Normal outcome		Adverse outcome				
Analyte	**Description**	**Function**	**Median**	**IQR**	**Median**	**IQR**	**ΔMFI**	**log₂ Fold Change**	***p****
SEZ6	Seizure protein 6	Neural growth and differentiation	558	489-637	383	317-438	**175**	0.5	2.93E-04
IL1A	Interleukin 1 alpha	Regulates immune responses	756	719-869	658	566-677	**98**	0.2	2.93E-04
C11orf87	Neural integral membrane protein 1	Ion transport in brain cells	630	595-786	499	372-581	**131**	0.3	3.67E-04
VEGFC	Vascular endothelial growth factor C	Promotes angiogenesis	524	490-566	414	353-443	**110**	0.3	3.67E-04
NKAIN2	Na+/K+ transporting ATPase interacting protein 2	Nervous system development	727	708-865	652	550-670	**75**	0.15	4.09E-04
HPCA	Hippocalcin	Neurogenesis and astrocytic differentiation regulation	484	442-539	415	337-427	**69**	0.2	6.30E-04
TGFB2	Transforming growth factor beta 2	Brain development and neuroprotection	738	711-835	646	542-677	**92**	0.2	7.01E-04
TBR1	Neuron-specific T-box transcription factor	Cortical development	806	780-914	730	642-783	**76**	0.14	7.01E-04
NCAN	Neurocan core protein	Neuronal migration and plasticity	667	633-791	501	413-566	**166.5**	0.4	7.79E-04
GPM6B	Glycoprotein M6B	Neuronal differentiation and myelination	693	641-788	617	540-640	**76**	0.16	7.79E-04
DIRAS2	GTP-binding RAS-like protein 2	Synaptic function in hippocampus and cerebral cortex	523	488-573	452	372-483	**72**	0.2	8.64E-04

Cerebrospinal fluid proteins exhibiting significant median fluorescent intensity differences between preterm infants with adverse outcome and preterm infants with normal outcome, at threshold of $p<0.001$, established by Mann-Whitney U test. Adverse outcome was defined as neuromotor delay, cerebral palsy, seizure disorder, mental developmental index < 85, deafness or blindness at 18-24 months assessment. Abbreviations; ΔMFI = median fluorescent intensity differences, IQR = inter quartile range. *p = <0.001.

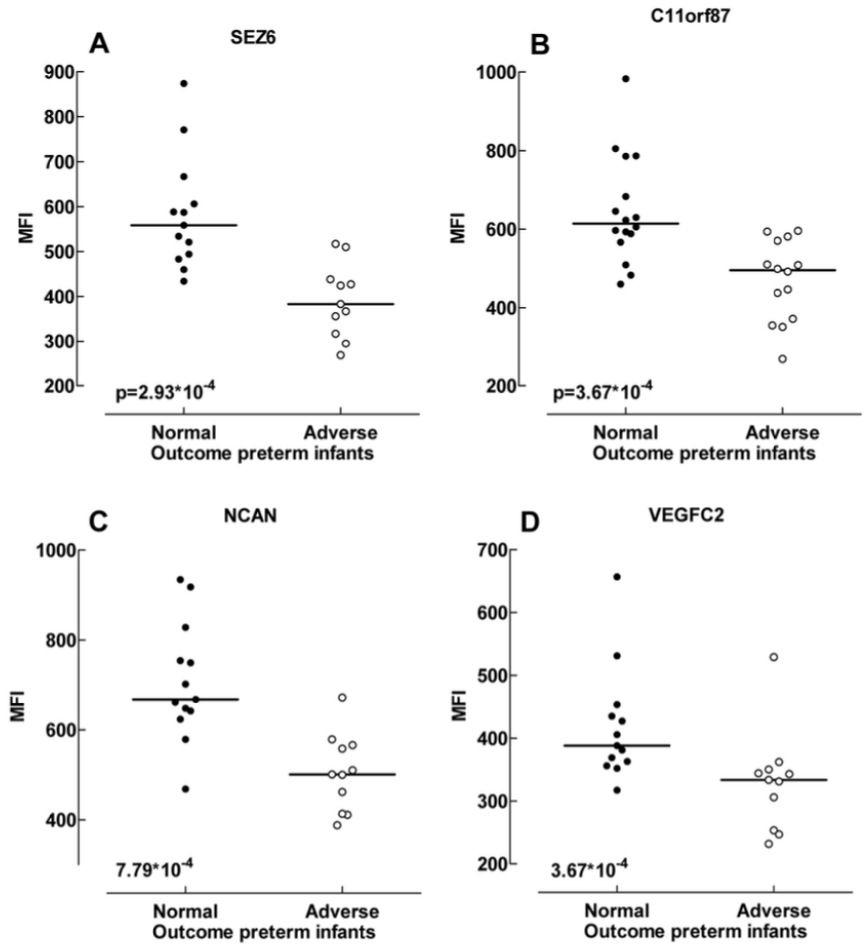

Figure 3. Protein differences between outcome groups
Scatter plot visualizing differences in median fluorescent intensity levels between outcome groups of preterm infants. Four of the proteins that exhibited the largest differences; Sez 6 (seizure protein 6) (**A**), C11orf87 (neuronal integral membrane protein 1) (**B**), NCAN (neurocan core protein) (**C**) and VEGFC (vascular endothelial growth factor C) (**D**). Adverse outcome was defined as neuromotor delay, cerebral palsy, seizure disorder, mental developmental index < 85, deafness or blindness at 18-24 months assessment. Data analyzed with Mann-Whitney U test, $p<0.001$.

DISCUSSION

We show a combination of inflammatory and neurodevelopmentally related CSF proteomic signatures in preterm infants in the neonatal period. Employing the sensitivity of a targeted antibody microarray assay, we identified increased concentrations of 35 proteins in preterm infants compared with term infants. Notably, adverse neurodevelopmental outcome in preterm infants correlated with lower protein levels.

Neuroinflammation and brain development

An upregulation of proteins associated with brain development, synaptic plasticity and neuroinflammation was observed in the preterm group compared with term infants. Proteins that differed the most were proteins associated with the complement system, including CFB, C5, and MASP2, and the inflammation regulating protein vascular cell adhesion molecule 1 (VCAM). Increased levels of inflammatory proteins in CSF, including the proteins of the complement cascade, have previously been associated with preterm birth (Boardman et al., 2018; Pataky, Howie, Girardi, & Boardman, 2017). Components of the complement system have been shown to be produced locally in the brain (Bellander, Singhrao, Ohlsson, Mattsson, & Svensson, 2001; Gorelik et al., 2017). They contribute to tissue damage in the injured or diseased brain (Hammad, Westacott, & Zaben, 2018). There is also evidence of the involvement of the complement system in brain development and altered neuroplasticity following postnatal events (Magdalon et al., 2020). In a mouse model of inflammation induced preterm birth, C5 was associated with decreased expression of neural markers and cortical abnormalities (Pedroni et al., 2014). However, opposing roles have been observed, and C5 is also associated with neuroprotection and normal neurodevelopment (Benard et al., 2008; Biggins, Brennan, Taylor, Woodruff, & Ruitenberg, 2017). Proliferation of neural progenitor cells was observed in mouse embryos treated with C5aR agonist (Coulthard et al., 2017). VCAM also has an important role in brain development. It is essential for the proliferation of neural stem cells in early development and the lack of VCAM leads to dysmaturation of neural progenitor cells (Hu et al., 2017).

Immunoregulatory proteins can have both detrimental and beneficial effects. Most of the proteins that were present in higher concentrations in preterm infants with normal outcome versus term controls seem to be involved in normal brain development (Boulanger, 2009). DSCAM is one of these proteins and was found higher in preterm infants, in comparison with term infants. It is involved in neurite guidance and synapse formation (Ly et al., 2008) as well as in the innate immune response. Increased concentrations of several other neurodevelopmentally related proteins were observed in the preterm population compared with term infants. This likely reflect the rapid neurodevelopment and growth during the early developmental stages of the preterm brain, which might explain in part the vulnerability of the preterm brain to a diversity of external stimuli. The data differences between preterm and term infants were even larger when only the preterm and term infants with normal outcome were compared. One of these was activin receptor type-1 (ACVR1), which is a transmembrane kinase receptor for members of the transforming growth factor-β (TGFB) family with neurotrophic and neuroprotective properties (Kupershmidt, Amit, Bar-Am, Youdim, & Blumenfeld, 2007; Suzuki, Kobayashi, Funatsu, Morita, & Ikekita, 2010). Moreover, several proteins primarily localized in synapsis and involved in cell interaction and synaptogenesis were found higher in the preterm population. These included CASKIN1, ARPP21 and amyloid beta precursor (APP), important post-transcriptional regulators of dendritic growth as well as synaptic formation and plasticity (Bencsik et al., 2019; Rehfeld et al., 2018). Recently, experimental studies suggested a correlation between the serine protein kinase CASKIN1 and stress respons and memory formation (Bencsik et al., 2019; Katano et al., 2018). Moreover, human mutations in CASKIN1 is present in neurodevelopment disorders and correlated to

presynaptic dysfunction (Becker et al., 2020). CASKIN1 alterations have in preclinical rodent models been seen to be responsible for the presentation of autism spectrum disorder (ASD) behavioral phenotypes (Daimon et al., 2015).
Culture results did not influence the observed protein differences and neither did we observe differences in C-reactive protein levels between the groups. Preterm infants might have increased susceptibility to severe disease partly as a result of naïve immune status and compromised responsiveness of immune system (Olin et al., 2018). Recently, distinct, and unified blood protein profiles were observed in preterm infants directly at birth while different trends of the infants' blood protein profiles were observed shortly after, depending on diverse postnatal events (Zhong et al., 2021). Differences in plasma protein profiles in preterm infants have also been correlated with changes in gestational age (Suski et al., 2018).
Few proteins were found in higher concentrations in the term group compared with the preterm infants. Of these, transmembrane protein 132D differed the most. This is a protein found in mature oligodendrocytes and therefore not yet fully expressed in the preterm infant's brain. Also, aquaporin-4 (AQP4) protein was higher in term than in preterm infants. AQP4 is a CSN water channel involved in part in the glymphatic CSF clearance system and its dysfunction has been correlated with cognitive decline in the elderly (Jessen et al., 2015; Kress et al., 2014). Whether lower levels of AQP4 in preterm infants indicate a less mature glymphatic system needs further evaluation. For the investigation of the glymphatic function in different conditions, simultaneous analysis of protein concentrations in peripheral blood and CSF would be preferable.

Markers of adverse neurodevelopmental outcome

It has been suggested that neuroinflammation may promote neurodevelopmental impairment by altering the expression of developmental genes (Hagberg et al., 2015; Kuban et al., 2019). However, the exact molecular pathways and mechanisms leading to adverse neurodevelopmental outcome is not fully understood. We have recently revealed how the proteomic profile after perinatal asphyxia can predict death and long-term disability in term infants. In that report we revealed several novel protein biomarkers, that were elevated in term infants with adverse outcome (Leifsdottir et al., 2022). In contrast to term infants, here lower CSF levels of several mediators of neurodevelopment predicted outcome of preterm infants. Recently, Zhong et al., observed trends of both up- and downregulations of protein profiles in 14 preterm infants, depending on exposure to different postnatal events (Zhong et al., 2021). In that study correlation of the plasma protein profiles to neurodevelopmental outcomes was not performed. However, decreased protein levels in serum of those same 14 infants were associated with severe retinopathy of prematurity (Danielsson et al., 2021). Retinopathy of prematurity is a disease that is associated with the lowest gestational ages and its severity increases with increasing prematurity. Both up- and downregulation of several mediators of neurodevelopment was shown in a study on preterm infants with post hemorrhagic ventricular dilatation, compared with healthy term infants (Morales et al., 2012). In that study several of the observed proteins were found in lower amounts after initiation of medical treatment. No correlation with clinical outcome was performed but post hemorrhagic ventricular dilatation is a condition that carries a high risk of neurodevelopmental adversities. In a recent study downregulation of neurotrophic proteins was correlated with lower brain volumes and later impaired cognitive development in preterm infants (Kuban et al., 2019). Thus, our data on CSF protein signatures, is in line with these studies. Moreover, lower levels of neurodevelopmental proteins associate with cognitive decline in the elderly (Harris et al., 2020; Ju, Xu, Wang, & Zhang, 2019).
The proteins that differed the most between outcome groups were regulatory proteins of brain development. These included vascular endothelial growth factor C (VEGFC), that promotes and regulates angiogenesis in brain both during development and repair. It has also been shown

to have trophical effects on neural progenitor cells (Le Bras et al., 2006). Serum VEGF concentrations of preterm infants are dynamic the first week of life (Brooks et al., 2021). Recently the administration of VEGFC was shown to improve neurological repair following traumatic brain injury in a rat model through modulating microglial activation (Ju et al., 2019). Thus, low levels in preterm infants are likely associated with inadequate angiogenesis and a hampered neurogenesis.

Neurocan core protein (NCAN) and TGFB levels also correlated with outcome. NCAN is a component of the extracellular matrix in the brain and has an important role in neuronal migration and plasticity, especially the development of the cortex (Schwartz & Domowicz, 2018). TGFB is a multifunctional cytokine that enhances the expression of NCAN (Asher et al., 2000). The lack of NCAN has been associated with cognitive decline and diminished brain volumes in the elderly (Harris et al., 2020). These findings are in contrast with findings of experimental study where upregulation of these proteins was observed in traumatically injured brain tissue and correlated with delayed axonal repair (Asher et al., 2000). These differences could be due to different underlying causes. The neurological adversities of preterm infants, like in the elderly, are in large part due to disturbances in brain development, while the sequelae of traumatic brain injury stem from lesions following an insult, and the formation of scar tissue.

Another mediator of cortical development and essential for cognitive development is neuron-specific T-box transcription factor (TBR1) (Vegas et al., 2018). This was one of the protein that was found at lower levels in preterm infants with adverse outcome compared with normal. Mutations in Tbr1 gene caused synaptic and neuronal dysfunctions that led to ASD like behavior (Yook et al., 2019). Preterm infants are at significantly higher risk at developing ASD than children born at term age (Agrawal, Rao, Bulsara, & Patole, 2018; Soul & Spence, 2020).

Seizure protein 6 (Sez6) and Na+/K+ transport ATPase interacting protein 2 (NKAIN2) are essential proteins in nervous systems' health and development. Lower levels of these proteins were correlated with adverse outcome of preterm infants in the present study. NKAIN2 is associated with myelination. Chromosomal deletion of the region responsible for this protein has been correlated with both cerebral atrophy and abnormal white matter development (Bocciardi et al., 2005; Zhao, Zhou, Luo, Mao, & Lu, 2015). The lack of Sez6 has been linked with impairment in motor functions, memory, and cognition in experimental studies (Nash et al., 2020). Another protein that was lower in the adverse outcome group was chromosome 11 open reading frame 87 (c11orf87) protein, also known as neuronal integral membrane protein 1. It is primarily expressed in brain tissue and suppressed levels have been correlated to schizophrenia related pathways (Etemadikhah, Niazi, Wetterberg, & Feuk, 2020). Notably, dysfunction of synaptic transmission, synaptic plasticity and behavior pathways in brain has been implicated in schizophrenia (Fromer et al., 2014). Preterm birth, especially before week 32, is associated with a 3-to-4-fold increased risk of developing neuropsychiatric disorders, including schizophrenia (Vanes, Murray, & Nosarti, 2021). To the best of our knowledge, we are the first to report relatively lower levels of c11orf87 protein in the preterm CSF. Future work could delineate whether its expression is transcriptionally or epigenetically regulated in the preterm brain.

Clinical implication

Preterm birth is associated with complex brain abnormalities resulting from immaturity. Early prediction and better understanding the mechanisms for neurodevelopmental outcome in this high-risk population is crucial. It would allow more individualized support to minimize neurological sequalae. Protein profile analysis is a methodology that provides insight into the biological processes that the proteins participate in. Thus, revealing changes in these processes, that might lead to aberrant neurodevelopment.

Although many questions are unresolved about the exact pathological process of developmental brain injury, neuroinflammation is an important contributor. Therefore,

inflammatory proteins in CSF are of clinical importance. Furthermore, many questions are yet to be answered about the role of the different neurodevelopmentally related proteins. The present study contributes to the knowledge of the physiological changes that occur in the developing brain as measured by markers in CSF. Thus, it may aid in the identification of infants at risk for adverse neurodevelopmental outcome. Therapeutic options for preterm brain injury are currently limited. The present study indicates that preterm infants with adverse outcome have lower CSF levels of several of the proteins critical for brain development, neuro- and synaptogenesis. This is in line with experimental studies in stroke models, where VEGFC treatments diminished brain injury and enhanced recovery (Dzietko, Derugin, Wendland, Vexler, & Ferriero, 2013). Notably, in other pathological conditions of premature birth, the blocking of proteins may enhance recovery, as has been established with anti-VEGF treatment in retinopathy of prematurity (VanderVeen et al., 2017).

Parenteral nutrition initiated earlier, with higher amounts of protein and fat improve the growth of extremely preterm infants including their head circumference (Morgan, McGowan, Herwitker, Hart, & Turner, 2014; Westin et al., 2018). We speculate that part of the mechanism is to provide the developing brain with crucial proteins enabling adequate brain development and better overall outcome. To provide enough macronutrient intake, including protein substrates, is a modifiable factor that could reduce the risk of adverse neurodevelopmental outcomes for these fragile infants.

Strengths and Limitations

The strengths of this study include the unique biobank of CSF from preterm and term infants, collected under clinical routine care. We also have performed an in-depth broad analysis of protein profiles in CSF, using a novel emerging technique to simultaneously evaluate the levels of 178 proteins in small samples. This enabled us to provide insight into pathophysiological changes that may lead to adverse neurodevelopment in preterm infants.

We acknowledge some limitations of the present study. All infants included in the study had clinical and laboratory signs of infection and a substantial part of the preterm group had positive cultures in blood and/or CSF (16/26). However, in the term group only infants with negative cultures were included. The age of the infants at the time of CSF collection varied, with the term group being significantly younger than the preterm group. The protein selection is inevitably biased as it was chosen based on previous studies. Many of the analysed proteins have correlations with infection and brain development. Also, we included proteins that have been studied in brain ischemia and other forms of brain trauma, for which more research is available in older children and adults. Another limitation is the assessment of neurodevelopmental outcome that was done in a non-standardized way. This is due to the fact, that follow-up was not a part of the study protocol, but was conducted according to current clinical routine. Therefore, it differed depending on degree of prematurity and on clinical findings. Nevertheless, all infants had a neurological evaluation in the time period from 18 to 24 moths, corrected age.

CONCLUSION

Numerous protein abundances in preterm infants in comparison with term infants, were demonstrated, the upregulation of proteins related to neuroinflammation being the most prominent. Preterm infants with unfavorable outcome shared a specific proteomic profile in CSF, with generally lower levels of functional and structural brain related proteins, in comparison with preterm infants with favourable outcome. This was independent of blood or CSF culture results. In the present study the CSF proteomic signatures of preterm infants observed here may reflect developmental changes in response to external or internal events and have value in predicting long-term outcome. The study contributes to the understanding of the

neurodevelopmental processes in the preterm brain. It renders novel data regarding essential proteins for optimal neurodevelopmental outcome and suggests a vulnerability towards the lak of these proteins. This upens up possibilities of tentative treatment options for preterm infants.

Ethics Statement

The regional ethical review board at the Karolinska Institute and Stockholm County (Dnr 98-246, 2003-174, 2011/1891-31) approved this study. It was carried out in accordance with European Community guidelines and the Declaration of Helsinki.

Author Contributions

KL, VS, ET and EH conceptualized and designed the study
KL, EH and VS enrolled the patients and collected the samples. KL, EH, KJ and VS collected and interpreted clinical data. PN and ET were responsible for sample analysis. KL, EH, ET and KJ analyzed the data. KL, PL and SE did statistical analysis. KL, ET, EH, KJ, ÁH and VS wrote the manuscript. All authors read and approved the final manuscript.

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Abbreviations

ACVR1; activin receptor type-1, APP; amyloid beta precursor, AQP4; aquaporin-4 ARPP21; cyclic AMP-regulated phosphoprotein 21, c11orf87; chromosome 11 open reading frame 87, ASD; autism spectrum disorder, CASKIN1; calcium/calmodulin-dependent serine protein kinase (CASK)-interacting protein-1, C5; complement 5, C5aR; C5a receptor C9; complement 9, CFB; complement factor B, CNS; central nervous system, CSF; cerebrospinal fluid, DSCAM; Down syndrome cell adhesion molecule, IQR; inter quartile range, MASP2; mannose-binding protein-associated serine protease 2, NCAN; Neurocan core protein, NETO1; Neurophilin and tolloid like proten 1, NKAIN2; Na+/K+ transport ATPase interacting protein 2, SEZ6; Seizure protein 6, TGFB; transforming growth factor-β family, VCAM1; vascular cell adhesion molecule1, VEGFC; vascular endothelial growth factor C.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

References

Agrawal, S., Rao, S. C., Bulsara, M. K., & Patole, S. K. (2018). Prevalence of Autism Spectrum Disorder in Preterm Infants: A Meta-analysis. *Pediatrics, 142*(3). doi:10.1542/peds.2018-0134

Asher, R. A., Morgenstern, D. A., Fidler, P. S., Adcock, K. H., Oohira, A., Braistead, J. E., . . . Fawcett, J. W. (2000). Neurocan is upregulated in injured brain and in cytokine-treated astrocytes. *J Neurosci, 20*(7), 2427-2438. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/10729323

Bayley, N. (1993). *Bayley Scales of Infant Development* (2nd ed.): Psychological Corporation.

Becker, M., Mastropasqua, F., Reising, J. P., Maier, S., Ho, M. L., Rabkina, I., . . . Tammimies, K. (2020). Presynaptic dysfunction in CASK-related neurodevelopmental disorders. *Transl Psychiatry, 10*(1), 312. doi:10.1038/s41398-020-00994-0

Bell, E. F., Hintz, S. R., Hansen, N. I., Bann, C. M., Wyckoff, M. H., DeMauro, S. B., . . . Human Development Neonatal Research, N. (2022). Mortality, In-Hospital Morbidity, Care Practices, and 2-Year Outcomes for Extremely Preterm Infants in the US, 2013-2018. *JAMA, 327*(3), 248-263. doi:10.1001/jama.2021.23580

Bellander, B. M., Singhrao, S. K., Ohlsson, M., Mattsson, P., & Svensson, M. (2001). Complement activation in the human brain after traumatic head injury. *J Neurotrauma, 18*(12), 1295-1311. doi:10.1089/08977150152725605

Benard, M., Raoult, E., Vaudry, D., Leprince, J., Falluel-Morel, A., Gonzalez, B. J., . . . Fontaine, M. (2008). Role of complement anaphylatoxin receptors (C3aR, C5aR) in the development of the rat cerebellum. *Mol Immunol, 45*(14), 3767-3774. doi:10.1016/j.molimm.2008.05.027

Bencsik, N., Pusztai, S., Borbely, S., Fekete, A., Dulk, M., Kis, V., . . . Schlett, K. (2019). Dendritic spine morphology and memory formation depend on postsynaptic Caskin proteins. *Sci Rep, 9*(1), 16843. doi:10.1038/s41598-019-53317-9

Biggins, P. J. C., Brennan, F. H., Taylor, S. M., Woodruff, T. M., & Ruitenberg, M. J. (2017). The Alternative Receptor for Complement Component 5a, C5aR2, Conveys Neuroprotection in Traumatic Spinal Cord Injury. *J Neurotrauma, 34*(12), 2075-2085. doi:10.1089/neu.2016.4701

Boardman, J. P., Ireland, G., Sullivan, G., Pataky, R., Fleiss, B., Gressens, P., & Miron, V. (2018). The Cerebrospinal Fluid Inflammatory Response to Preterm Birth. *Front Physiol, 9*, 1299. doi:10.3389/fphys.2018.01299

Bocciardi, R., Giorda, R., Marigo, V., Zordan, P., Montanaro, D., Gimelli, S., . . . Gimelli, G. (2005). Molecular characterization of a t(2;6) balanced translocation that is associated with a complex phenotype and leads to truncation of the TCBA1 gene. *Hum Mutat, 26*(5), 426-436. doi:10.1002/humu.20235

Boulanger, L. M. (2009). Immune proteins in brain development and synaptic plasticity. *Neuron, 64*(1), 93-109. doi:10.1016/j.neuron.2009.09.001

Brooks, S., Friedes, B. D., Northington, F., Graham, E., Tekes, A., Burton, V. J., . . . Everett, A. D. (2021). Serum brain injury biomarkers are gestationally and post-natally regulated in non-brain injured neonates. *Pediatr Res*. doi:10.1038/s41390-021-01906-8

Chau, V., Brant, R., Poskitt, K. J., Tam, E. W., Synnes, A., & Miller, S. P. (2012). Postnatal infection is associated with widespread abnormalities of brain development in premature newborns. *Pediatr Res, 71*(3), 274-279. doi:10.1038/pr.2011.40

Chau, V., Synnes, A., Grunau, R. E., Poskitt, K. J., Brant, R., & Miller, S. P. (2013). Abnormal brain maturation in preterm neonates associated with adverse developmental outcomes. *Neurology, 81*(24), 2082-2089. doi:10.1212/01.wnl.0000437298.43688.b9

Cheong, J. L. Y., Olsen, J. E., Lee, K. J., Spittle, A. J., Opie, G. F., Clark, M., . . . Victorian Infant Collaborative Study, G. (2021). Temporal Trends in Neurodevelopmental Outcomes to 2 Years After Extremely Preterm Birth. *JAMA Pediatr, 175*(10), 1035-1042. doi:10.1001/jamapediatrics.2021.2052

Collaborators. (2021, November 18). Human protein atlas. Retrieved from https://www.proteinatlas.org/

Coulthard, L. G., Hawksworth, O. A., Li, R., Balachandran, A., Lee, J. D., Sepehrband, F., . . . Woodruff, T. M. (2017). Complement C5aR1 Signaling Promotes Polarization and Proliferation of Embryonic Neural Progenitor Cells through PKCzeta. *J Neurosci, 37*(22), 5395-5407. doi:10.1523/JNEUROSCI.0525-17.2017

Daimon, C. M., Jasien, J. M., Wood, W. H., 3rd, Zhang, Y., Becker, K. G., Silverman, J. L., . . . Maudsley, S. (2015). Hippocampal Transcriptomic and Proteomic Alterations in the BTBR Mouse Model of Autism Spectrum Disorder. *Front Physiol, 6*, 324. doi:10.3389/fphys.2015.00324

Danielsson, H., Tebani, A., Zhong, W., Fagerberg, L., Brusselaers, N., Hard, A. L., . . . Hellstrom, A. (2021). Blood protein profiles related to preterm birth and retinopathy of prematurity. *Pediatr Res*. doi:10.1038/s41390-021-01528-0

Dzietko, M., Derugin, N., Wendland, M. F., Vexler, Z. S., & Ferriero, D. M. (2013). Delayed VEGF treatment enhances angiogenesis and recovery after neonatal focal rodent stroke. *Transl Stroke Res, 4*(2), 189-200. doi:10.1007/s12975-012-0221-6

Ellison, V. J., Mocatta, T. J., Winterbourn, C. C., Darlow, B. A., Volpe, J. J., & Inder, T. E. (2005). The relationship of CSF and plasma cytokine levels to cerebral white matter injury in the premature newborn. *Pediatr Res, 57*(2), 282-286. doi:10.1203/01.PDR.0000148286.53572.95

Etemadikhah, M., Niazi, A., Wetterberg, L., & Feuk, L. (2020). Transcriptome analysis of fibroblasts from schizophrenia patients reveals differential expression of schizophrenia-related genes. *Sci Rep, 10*(1), 630. doi:10.1038/s41598-020-57467-z

Fromer, M., Pocklington, A. J., Kavanagh, D. H., Williams, H. J., Dwyer, S., Gormley, P., . . . O'Donovan, M. C. (2014). De novo mutations in schizophrenia implicate synaptic networks. *Nature, 506*(7487), 179-184. doi:10.1038/nature12929

Gorelik, A., Sapir, T., Haffner-Krausz, R., Olender, T., Woodruff, T. M., & Reiner, O. (2017). Developmental activities of the complement pathway in migrating neurons. *Nat Commun, 8*, 15096. doi:10.1038/ncomms15096

Gozdas, E., Parikh, N. A., Merhar, S. L., Tkach, J. A., He, L., & Holland, S. K. (2018). Altered functional network connectivity in preterm infants: antecedents of cognitive and motor impairments? *Brain Struct Funct, 223*(8), 3665-3680. doi:10.1007/s00429-018-1707-0

Hagberg, H., Mallard, C., Ferriero, D. M., Vannucci, S. J., Levison, S. W., Vexler, Z. S., & Gressens, P. (2015). The role of inflammation in perinatal brain injury. *Nat Rev Neurol, 11*(4), 192-208. doi:10.1038/nrneurol.2015.13

Haggmark, A., Bystrom, S., Ayoglu, B., Qundos, U., Uhlen, M., Khademi, M., . . . Nilsson, P. (2013). Antibody-based profiling of cerebrospinal fluid within multiple sclerosis. *Proteomics, 13*(15), 2256-2267. doi:10.1002/pmic.201200580

Hammad, A., Westacott, L., & Zaben, M. (2018). The role of the complement system in traumatic brain injury: a review. *J Neuroinflammation, 15*(1), 24. doi:10.1186/s12974-018-1066-z

Harris, S. E., Cox, S. R., Bell, S., Marioni, R. E., Prins, B. P., Pattie, A., . . . Deary, I. J. (2020). Neurology-related protein biomarkers are associated with cognitive ability and brain volume in older age. *Nat Commun, 11*(1), 800. doi:10.1038/s41467-019-14161-7

Hu, X. L., Chen, G., Zhang, S., Zheng, J., Wu, J., Bai, Q. R., . . . Shen, Q. (2017). Persistent Expression of VCAM1 in Radial Glial Cells Is Required for the Embryonic Origin of Postnatal Neural Stem Cells. *Neuron, 95*(2), 309-325 e306. doi:10.1016/j.neuron.2017.06.047

Jessen, N. A., Munk, A. S., Lundgaard, I., & Nedergaard, M. (2015). The Glymphatic System: A Beginner's Guide. *Neurochem Res, 40*(12), 2583-2599. doi:10.1007/s11064-015-1581-6

Ju, S., Xu, C., Wang, G., & Zhang, L. (2019). VEGF-C Induces Alternative Activation of Microglia to Promote Recovery from Traumatic Brain Injury. *J Alzheimers Dis, 68*(4), 1687-1697. doi:10.3233/JAD-190063

Katano, T., Takao, K., Abe, M., Yamazaki, M., Watanabe, M., Miyakawa, T., . . . Ito, S. (2018). Distribution of Caskin1 protein and phenotypic characterization of its knockout mice using a comprehensive behavioral test battery. *Mol Brain, 11*(1), 63. doi:10.1186/s13041-018-0407-2

Kress, B. T., Iliff, J. J., Xia, M., Wang, M., Wei, H. S., Zeppenfeld, D., . . . Nedergaard, M. (2014). Impairment of paravascular clearance pathways in the aging brain. *Ann Neurol, 76*(6), 845-861. doi:10.1002/ana.24271

Kuban, K. C. K., Jara, H., O'Shea, T. M., Heeren, T., Joseph, R. M., Fichorova, R. N., . . . Investigators, E. S. (2019). Association of Circulating Proinflammatory and Anti-inflammatory Protein Biomarkers in Extremely Preterm Born Children with Subsequent Brain Magnetic Resonance Imaging Volumes and Cognitive Function at Age 10 Years. *J Pediatr, 210*, 81-90 e83. doi:10.1016/j.jpeds.2019.03.018

Kupershmidt, L., Amit, T., Bar-Am, O., Youdim, M. B., & Blumenfeld, Z. (2007). The neuroprotective effect of Activin A and B: implication for neurodegenerative diseases. *J Neurochem, 103*(3), 962-971. doi:10.1111/j.1471-4159.2007.04785.x

Le Bras, B., Barallobre, M. J., Homman-Ludiye, J., Ny, A., Wyns, S., Tammela, T., . . . Thomas, J. L. (2006). VEGF-C is a trophic factor for neural progenitors in the vertebrate embryonic brain. *Nat Neurosci, 9*(3), 340-348. doi:10.1038/nn1646

Leifsdottir, K., Thelin, E. P., Lassaren, P., Siljehav, V., Nilsson, P., Eksborg, S., & Herlenius, E. (2022). Proteomic profiles in cerebrospinal fluid predicted death and disability in term infants with perinatal asphyxia: A pilot study. *Acta Paediatr*. doi:10.1111/apa.16277

Lindblad, C., Pin, E., Just, D., Al Nimer, F., Nilsson, P., Bellander, B. M., . . . Thelin, E. P. (2021). Fluid proteomics of CSF and serum reveal important neuroinflammatory proteins in blood-brain barrier disruption and outcome prediction following severe traumatic brain injury: a prospective, observational study. *Crit Care, 25*(1), 103. doi:10.1186/s13054-021-03503-x

Ly, A., Nikolaev, A., Suresh, G., Zheng, Y., Tessier-Lavigne, M., & Stein, E. (2008). DSCAM is a netrin receptor that collaborates with DCC in mediating turning responses to netrin-1. *Cell, 133*(7), 1241-1254. doi:10.1016/j.cell.2008.05.030

Magdalon, J., Mansur, F., Teles, E. S. A. L., de Goes, V. A., Reiner, O., & Sertie, A. L. (2020). Complement System in Brain Architecture and Neurodevelopmental Disorders. *Front Neurosci, 14*, 23. doi:10.3389/fnins.2020.00023

Mallard, C., & Hagberg, H. (2007). Inflammation-induced preconditioning in the immature brain. *Semin Fetal Neonatal Med, 12*(4), 280-286. doi:10.1016/j.siny.2007.01.014

Mathur, A., & Inder, T. (2009). Magnetic resonance imaging--insights into brain injury and outcomes in premature infants. *J Commun Disord, 42*(4), 248-255. doi:10.1016/j.jcomdis.2009.03.007

Morales, D. M., Townsend, R. R., Malone, J. P., Ewersmann, C. A., Macy, E. M., Inder, T. E., & Limbrick, D. D., Jr. (2012). Alterations in protein regulators of neurodevelopment in the cerebrospinal fluid of infants with posthemorrhagic hydrocephalus of prematurity. *Mol Cell Proteomics, 11*(6), M111 011973. doi:10.1074/mcp.M111.011973

Morgan, C., McGowan, P., Herwitker, S., Hart, A. E., & Turner, M. A. (2014). Postnatal head growth in preterm infants: a randomized controlled parenteral nutrition study. *Pediatrics, 133*(1), e120-128. doi:10.1542/peds.2013-2207

Mullen, K. M., Vohr, B. R., Katz, K. H., Schneider, K. C., Lacadie, C., Hampson, M., . . . Ment, L. R. (2011). Preterm birth results in alterations in neural connectivity at age 16 years. *Neuroimage, 54*(4), 2563-2570. doi:10.1016/j.neuroimage.2010.11.019

Nash, A., Aumann, T. D., Pigoni, M., Lichtenthaler, S. F., Takeshima, H., Munro, K. M., & Gunnersen, J. M. (2020). Lack of Sez6 Family Proteins Impairs Motor Functions, Short-Term Memory, and Cognitive Flexibility and Alters Dendritic Spine Properties. *Cerebral Cortex, 30*(4), 2167-2184. doi:10.1093/cercor/bhz230

Olin, A., Henckel, E., Chen, Y., Lakshmikanth, T., Pou, C., Mikes, J., . . . Brodin, P. (2018). Stereotypic Immune System Development in Newborn Children. *Cell, 174*(5), 1277-1292 e1214. doi:10.1016/j.cell.2018.06.045

Papile, L. A., Burstein, J., Burstein, R., & Koffler, H. (1978). Incidence and evolution of subependymal and intraventricular hemorrhage: a study of infants with birth weights less than 1,500 gm. *J Pediatr, 92*(4), 529-534. doi:10.1016/s0022-3476(78)80282-0

Pataky, R., Howie, F. A., Girardi, G., & Boardman, J. P. (2017). Complement C5a is present in CSF of human newborns and is elevated in association with preterm birth. *J Matern Fetal Neonatal Med, 30*(20), 2413-2416. doi:10.1080/14767058.2016.1251896

Pedroni, S. M., Gonzalez, J. M., Wade, J., Jansen, M. A., Serio, A., Marshall, I., . . . Girardi, G. (2014). Complement inhibition and statins prevent fetal brain cortical abnormalities in a mouse model of preterm birth. *Biochim Biophys Acta, 1842*(1), 107-115. doi:10.1016/j.bbadis.2013.10.011

Pin, E., Sjoberg, R., Andersson, E., Hellstrom, C., Olofsson, J., Jernbom Falk, A., . . . Manberg, A. (2019). Array-Based Profiling of Proteins and Autoantibody Repertoires in CSF. *Methods Mol Biol, 2044*, 303-318. doi:10.1007/978-1-4939-9706-0_19

Rasmussen, M. K., Mestre, H., & Nedergaard, M. (2018). The glymphatic pathway in neurological disorders. *Lancet Neurol, 17*(11), 1016-1024. doi:10.1016/S1474-4422(18)30318-1

Rehfeld, F., Maticzka, D., Grosser, S., Knauff, P., Eravci, M., Vida, I., . . . Wulczyn, F. G. (2018). The RNA-binding protein ARPP21 controls dendritic branching by functionally opposing the miRNA it hosts. *Nat Commun, 9*(1), 1235. doi:10.1038/s41467-018-03681-3

Schwartz, N. B., & Domowicz, M. S. (2018). Proteoglycans in brain development and pathogenesis. *FEBS Lett, 592*(23), 3791-3805. doi:10.1002/1873-3468.13026

Schwenk, J. M., Gry, M., Rimini, R., Uhlen, M., & Nilsson, P. (2008). Antibody suspension bead arrays within serum proteomics. *J Proteome Res, 7*(8), 3168-3179. doi:10.1021/pr700890b

Soul, J. S., & Spence, S. J. (2020). Predicting Autism Spectrum Disorder in Very Preterm Infants. *Pediatrics, 146*(4). doi:10.1542/peds.2020-019448

Suski, M., Bokiniec, R., Szwarc-Duma, M., Madej, J., Bujak-Gizycka, B., Borszewska-Kornacka, M. K., . . . Kwinta, P. (2018). Plasma proteome changes in cord blood samples from preterm infants. *J Perinatol, 38*(9), 1182-1189. doi:10.1038/s41372-018-0150-7

Suzuki, K., Kobayashi, T., Funatsu, O., Morita, A., & Ikekita, M. (2010). Activin A induces neuronal differentiation and survival via ALK4 in a SMAD-independent manner in a

subpopulation of human neuroblastomas. *Biochem Biophys Res Commun, 394*(3), 639-645. doi:10.1016/j.bbrc.2010.03.039

Thompson, D. K., Kelly, C. E., Chen, J., Beare, R., Alexander, B., Seal, M. L., . . . Spittle, A. J. (2019). Characterisation of brain volume and microstructure at term-equivalent age in infants born across the gestational age spectrum. *Neuroimage Clin, 21*, 101630. doi:10.1016/j.nicl.2018.101630

VanderVeen, D. K., Melia, M., Yang, M. B., Hutchinson, A. K., Wilson, L. B., & Lambert, S. R. (2017). Anti-Vascular Endothelial Growth Factor Therapy for Primary Treatment of Type 1 Retinopathy of Prematurity: A Report by the American Academy of Ophthalmology. *Ophthalmology, 124*(5), 619-633. doi:10.1016/j.ophtha.2016.12.025

Vanes, L. D., Murray, R. M., & Nosarti, C. (2021). Adult outcome of preterm birth: Implications for neurodevelopmental theories of psychosis. *Schizophr Res*. doi:10.1016/j.schres.2021.04.007

Vegas, N., Cavallin, M., Kleefstra, T., de Boer, L., Philbert, M., Maillard, C., . . . Bahi-Buisson, N. (2018). Mutations in TBR1 gene leads to cortical malformations and intellectual disability. *Eur J Med Genet, 61*(12), 759-764. doi:10.1016/j.ejmg.2018.09.012

Westin, V., Klevebro, S., Domellof, M., Vanpee, M., Hallberg, B., & Stoltz Sjostrom, E. (2018). Improved nutrition for extremely preterm infants - A population based observational study. *Clin Nutr ESPEN, 23*, 245-251. doi:10.1016/j.clnesp.2017.09.004

Yook, C., Kim, K., Kim, D., Kang, H., Kim, S. G., Kim, E., & Kim, S. Y. (2019). A TBR1-K228E Mutation Induces Tbr1 Upregulation, Altered Cortical Distribution of Interneurons, Increased Inhibitory Synaptic Transmission, and Autistic-Like Behavioral Deficits in Mice. *Front Mol Neurosci, 12*, 241. doi:10.3389/fnmol.2019.00241

Zhao, S. C., Zhou, B. W., Luo, F., Mao, X., & Lu, Y. J. (2015). The structure and function of NKAIN2-a candidate tumor suppressor. *Int J Clin Exp Med, 8*(10), 17072-17079. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/26770299

Zhong, W., Danielsson, H., Tebani, A., Karlsson, M. J., Elfvin, A., Hellgren, G., . . . Uhlen, M. (2021). Dramatic changes in blood protein levels during the first week of life in extremely preterm infants. *Pediatr Res, 89*(3), 604-612. doi:10.1038/s41390-020-0912-8