Study of inflammatory markers in near-term and term neonates with neonatal encephalopathy treated with hypothermia +/- sildenafil

Ruofan Song, BSc

Integrated Program in Neuroscience

McGill University, Montreal

July 2022

A thesis submitted to McGill University

in partial fulfillment of the requirements of the degree of

Master of Science

TABLE OF CONTENTS

Abstract

Background: Presently, neonatal encephalopathy (NE) has only one proven treatment, therapeutic hypothermia (TH). However, many treated neonates still develop brain injury. Neuroinflammation is an important contributor in the pathophysiology of NE. Mitigating inflammation appears an interesting venue for new adjunctive treatments, and sildenafil has shown inflammation-modulating effects in rat models of NE.

Objective: This thesis aimed to further investigate the expression over the first days of life of blood inflammatory biomarkers in neonates with NE treated with TH +/- sildenafil.

Methods: Plasma samples were collected at different time-points during the first days of life (day 2, day 3, and day 4) in human neonates with NE with/without brain injury treated with TH +/- sildenafil; and inflammatory markers were quantified in these samples by multi-analyte profiling antigen analysis.

Results: When comparing neonates with NE treated with TH with and without brain injury on day 2 of life, there was an up-regulation of interleukin (IL)-12p40, stem cell factor (SCF), eotaxin-1, and pulmonary and activation-regulated chemokine (PARC) in neonates with brain injury compared to those without, suggesting a potential association between these markers and brain injury development in neonates with NE treated with TH. When comparing neonates with NE treated with TH +/- sildenafil over time during the first days of life, brain injury and/or sildenafil appeared to increase the expressions of cytokines interleukin-1 receptor antagonist (IL-1Ra) and IL-12p40, and chemokine eotaxin-1, while reducing the expression of inflammation mediator alpha-2-macroglobulin (A2Macro).

Conclusion: Elevated blood inflammatory protein levels were found associated with brain injury in neonates with NE treated with TH. Sildenafil in addition to TH appeared to exert beneficial

effects in these neonates with brain injury by modulating inflammation and potentially promoting neurorestoration, and may thus represent a promising adjunctive treatment to TH in the context of NE.

Résumé

Contexte: À l'heure actuelle, l'encéphalopathie néonatale (EN) n'a qu'un seul traitement prouvé, l'hypothermie thérapeutique (HT). Cependant, de nombreux nouveau-nés traités développent encore des lésions cérébrales. La neuroinflammation est un contributeur reconnu à la physiopathologie de l'EN. L'atténuation de l'inflammation semble être un mécanisme intéressant à cibler pour des traitements additionnels, et le sildénafil a montré des effets modulateur de l'inflammation dans des modèles de rat d'EN.

Objectif: Cette thèse vise à étudier l'expression au cours des premiers jours de vie de biomarqueurs inflammatoires sanguins chez les nouveau-nés atteints d'EN traités par HT +/- sildénafil.

Méthodes: Des échantillons de plasma ont été prélevés à différents moments au cours des premiers jours de vie (jour 2, jour 3 et jour 4) chez des nouveau-nés humains souffrant d'EN traités avec HT +/- sildénafil avec et sans lésion cérébrale; et des marqueurs inflammatoires ont été mesurés chez ces nouveau-nés par analyse antigénique de profilage multi-analytes.

Résultats: En comparant les nouveau-nés avec EN traités avec HT avec et sans lésion cérébrale au jour 2 de la vie, il y avait une régulation à la hausse de l'interleukine (IL)-12p40, du facteur de cellules souches (SCF), de l'éotaxine-1 et de l'activation pulmonaire et chimiokine régulée (PARC) chez les nouveau-nés atteints de lésions cérébrales par rapport à ceux sans, suggérant une association potentielle entre ces marqueurs et le développement de lésions cérébrales chez les nouveau-nés atteints d'EN traités par HT. Lors de la comparaison de nouveau-nés avec EN traités par HT +/- sildénafil au cours des premiers jours de vie, les lésions cérébrales et/ou le sildénafil semblaient augmenter l'expressions des cytokines antagonistes des récepteurs de

l'interleukine-1 (IL-1Ra) et IL-12p40, et de la chimiokine éotaxine-1, tout en réduisant l'expression du médiateur de l'inflammation alpha-2-macroglobuline (A2Macro).

Conclusion: Des taux élevés de protéines inflammatoires dans le sang ont été trouvés associés à des lésions cérébrales chez les nouveau-nés atteints d'EN traités par HT. Le sildénafil en plus de la HT semble exercer des effets bénéfiques chez ces nouveau-nés atteints de lésions cérébrales en modulant l'inflammation et en favorisant potentiellement la neurorestauration, et pourrait donc représenter un traitement prometteur en plus de l'HT dans l'EN.

Acknowledgements

I would like to express my deepest gratitude to my supervisor, Dr. Pia Wintermark, who offered me tremendous support and provided me with generous opportunities throughout my study. I could not have undertaken this journey without her invaluable advices, patient guidance, and continuous encouragement. I am truly grateful that Dr. Wintermark has always been understanding, and mentored me in a way that best-suited me.

I am also deeply grateful for my advisory committee members Dr. Guillaume Sébire and Dr. Jo Anne Stratton, who generously provided knowledgeable advices and exceptional supports. I would like to extend my thanks to my mentor Dr. Jean-Francois Cloutier for guiding me when I first started my graduate study and for holding my thesis seminar.

I wish to express my sincere appreciation and respect for all the neonates and their families included in this study for their trust, and all the healthcare professionals involved for their help. Many thanks to Dr. Christine Saint-Martin, who reviewed the brain magnetic resonance imaging and provided reports describing the brain injury.

This project would not have been possible without the assistance from my dearest current and past lab colleagues. I am very thankful for Ms. Aliona Fezoua and Mr. Daniel Ducharme who helped obtain the brain magnetic resonance imaging; Ms. Alexandra Belanger, Dr. Armin Yazdani, Mr. Belal Howidi, Mr. Eric Hongbo Xu, Ms. Palig Balian, Ms. Rana Ghafouri-Azar, and Ms. Zoë Ward who helped collect the plasma samples; Ms. Louise Merly, and Ms. Priscille-Nice Sanon who provided assistance in collecting patient data from the hospital database; Ms. Zehra Khoja who assisted with the shipment of the collected plasma samples to Rules-Based Medicine; and Ms. Alishia Poccia, who assisted in both collecting patient data and the shipment of plasma samples.

Last but not least, I want to thank my parents Ms. Wang Ge and Mr. Lijun Song for their unconditional love and support, without whom I could not be accomplishing my study. I want to thank my forever inspiration source, my grandfather Mr. Aiming Ge, who never stops learning and improving even now in his 80s. Special thanks to my sworn friends Ms. Chloe Chang and Ms. Karine Song for always standing by my side, and my best vent buddy Ms. Xiaoting You who provided me great emotional support.

Contribution of Authors

Ms. Ruofan Song, the author of this thesis, assisted in obtaining the brain magnetic resonance imaging in neonates with NE treated with TH; collected patient data from the hospital database; performed plasma sample collections; organized the shipment of the collected plasma samples to Rules-Based Medicine; designed and performed the data analysis; and wrote the thesis.

Dr. Pia Wintermark designed the study, supervised Ms. Ruofan Song and revised the thesis.

Dr. Emmanouil Rampakakis provided assistance for the statistical analysis.

List of Abbreviations

Aβ	β-amyloid
AD	Alzheimer's disease
aEEG	Amplitude-integrated electroencephalogram
ATP	Adenosine triphosphate
A2Macro	Alpha-2-macroglobulin
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
BG/W	Barkovich MRI basal ganglia/watershed score
B2M	Beta-2-microglobulin
CBF	Cerebral blood flow
CCL	C-C motif chemokine ligand
cGMP	Cyclic guanosine monophosphate
CNS	Central nervous system
CP	Cerebral palsy
CSF	Cerebrospinal fluid
DOL	Day of life
FTRN	Ferritin
G-CSF	Granulocyte-colony stimulating factor
GFAP	Glial fibrillary acidic protein
HI	Hypoxic-ischemic
HIE	Hypoxic-ischemic encephalopathy
ICAM-1	Intercellular adhesion molecule-1

IFN	Interferon
IL	Interleukin
IL-1Ra	Interleukin-1 receptor antagonist
LLOQ	Lower limit of quantification
LPS	Lipopolysaccharide
MAP	Multi-analyte profiling
MAPK	Mitogen-activated protein kinase
MCP	Monocyte chemoattractant protein
MIP	Macrophage inflammatory protein
MMP	Matrix metalloproteinase
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
MS	Multiple sclerosis
NDMA	N-methyl-D-aspartate
NE	Neonatal encephalopathy
NFκB	Nuclear factor-κB
NPC	Neural progenitor cell
P	Postnatal day
PAI-1	Plasminogen activator inhibitor-1
PARC	Pulmonary and activation-regulated chemokine
PDE5	Phosphodiesterase type 5
PKG	Phosphokinase G
QNS	Quantity not sufficient

RANTES	Regulated on activation, normal T cell expressed and secreted
ROS	Reactive oxygen species
SCF	Stem cell factor
SCI	Spinal cord injury
STAT3	Signal transducer and activator of transcription-3
TBI	Traumatic brain injury
TGF	Transforming growth factor
TH	Therapeutic hypothermia
Th	T helper cell
TIMP-1	Tissue inhibitor of metalloproteinases-1
TNF	Tumor necrosis factor
TNFR2	Tumor necrosis factor receptor 2
USP	United States Pharmacopeia
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
WBC	White blood cell

Introduction

Neonatal encephalopathy (NE) as a result of birth asphyxia is a condition characterized by an abnormal neurological function caused by a deprivation of oxygen (hypoxia) and/or blood (ischemia) to a neonate around the time of birth (Aslam et al., 2019; Hassell et al., 2015; Perlman, 1997; Shalak & Perlman, 2004; Volpe J. J., 2012). It has an incidence of 2 to 8 per 1000 live births in high-income countries (Lee et al., 2013) and remains a major cause of mortality and long-term disability in human neonates worldwide (Bryce et al., 2005). Neuroinflammation is one of the recognized pathophysiological contributor to NE, present from its acute phase to its longer-term phase (Ahearne et al., 2017; Chakkarapani et al., 2021; Fleiss & Gressens, 2012; Nair & Kumar, 2018). Inflammatory biomarkers in biological fluids have been extensively studied in clinical settings for their association with diagnosis, severity and/or outcome in neonates with NE (Ahearne et al., 2017; Bartha et al., 2004; Chalak et al., 2014; McGowan et al., 2021; Sävman et al., 1998; Walsh et al., 2013; Vasiljevic et al., 2011), and have also been utilized as an assessment tool for testing the efficacy of therapeutic approaches (Massaro et al., 2018; Orrock et al., 2016).

To date, the only proven treatment for moderate to severe NE is therapeutic hypothermia (TH) that includes cooling the whole body to a temperature of 33.5 ± 0.5°C initiated within 6 hours of birth and continued for 72 hours, followed by a slow rewarming until back to normothermia (Shankaran, 2012; Wintermark, 2011). However, TH does not confer neuroprotection in all cases, and many treated neonates still develop brain injury (Azzopardi et al., 2009; Chalak et al., 2014; Cheong et al., 2012; Gluckman et al., 2005; Wintermark, 2011). The efficacy of TH is limited by the timing of TH initiation and concomitant presence of inflammation in neonates with NE (Davidson et al., 2018; Gunn, A. J., & Gunn, T. R., 1998;

Nair & Kumar, 2018; Osredkar et al., 2014; Pauliah et al., 2013; Wintermark et al., 2010). In addition, TH is solely neuroprotective and does not repair brain injuries once they occurred (Chakkarapani et al., 2021; Gluckman et al., 2005; Shankaran et al., 2005; Yazdani et al., 2016). Therefore, there is an urgent need to find adjunctive therapies to complement TH that may improve the outcomes of neonates with NE. Attenuating inflammation appears an interesting venue for new adjunctive treatments, considering the important role of inflammation in the pathogenesis of NE (Aslam et al., 2019); and mitigating inflammation appears to lead to better neurological outcome in the context of NE (Jenkins et al., 2012).

Sildenafil is a promising potential adjunctive treatment. It is a potent phosphodiesterase type 5 (PDE5) inhibitor that increases the intracellular level of cyclic guanosine monophosphate (cGMP), which may inhibit neuroinflammation (Chakkarapani et al., 2021; Ghofrani et al., 2006; Peixoto et al., 2015). Sildenafil has been reported to have anti-inflammatory effects by reducing astrocyte and microglia activation, and by modulating inflammatory cytokines in animal models of adult neurological diseases and in rat models of NE (Araújo et al., 2020; Charriaut-Marlangue et al., 2014; Nunes et al., 2015; Yazdani et al., 2021; Zhang et al., 2013). It has been tested in adult patients with stroke (Silver et al., 2009), and is already used safely in neonates with persistent pulmonary hypertension (Simonca & Tulloh, 2017). The impact of sildenafil on inflammation in human neonates with NE is yet to be determined.

We hypothesize that inflammatory biomarkers may provide early guidance for therapeutic interventions in the context of NE, and sildenafil may keep its inflammation-modulating effects in human neonates with NE treated with TH. In the first aim of this thesis, we explored inflammatory biomarkers that are associated with brain injury in neonates with NE

treated with TH (Chapter 2). In the second aim of this thesis, we evaluated the impact of brain injury +/- sildenafil on inflammatory markers in early days of NE (Chapter 3).

Chapter 1 Review of the Relevant Literature

1.1 Neonatal encephalopathy

Neonatal encephalopathy (NE), also known as hypoxic-ischemic encephalopathy (HIE), is a condition with abnormal neurological function in a newly born infant, manifested by subnormal level of consciousness, depressed tone and reflexes, inability to initiate or maintain respiration, and/or seizures (Aoki et al., 2021; Aslam et al., 2019; Sarnat & Sarnat, 1976; Volpe J. J., 2012). The predominant etiologic factor of NE is birth asphyxia (Aslam et al., 2019; Hassell et al., 2015; Volpe J. J., 2012), which can occur due to a variety of antepartum, intrapartum, and post-natal events, such as placental abruption, uterine rupture, and cord prolapse (Gillam-Krakauer & Gowen, 2021). NE as a result of birth asphyxia has an estimated incidence of 2 to 8 per 1000 live births in high-income countries (Lee et al., 2013) and remains a leading cause (i.e., 23%) of neonatal mortality worldwide (Bryce et al., 2005). Moreover, children who survive birth asphyxia suffer from significant long-term morbidities, such as cerebral palsy, motor impairments, intellectual disabilities, and learning difficulties (Al-Macki et al., 2009; Wintermark, 2011).

1.2 Pathophysiology of neonatal encephalopathy (NE)

NE is an evolving process that involves three phases of brain damage (Chakkarapani et al., 2021; Davidson et al., 2021). Much of our current understanding of the pathogenic mechanisms in NE is from experiments in animal models (rats, mice, lambs, and piglets) (Grow

& Barks, 2002; Roohey et al., 1997), and some clinical investigations in human neonates (Johnston M. V., 2001; Wyatt et al., 1989).

1.2.1 Primary phase

The primary pathophysiological event that initiates NE is the deprivation of oxygen (hypoxia) and/or the impairment of blood flow (ischemia) (Perlman, 1997; Shalak & Perlman, 2004). The lack of oxygen and glucose gives rise to the primary energy failure characterized by decreased adenosine triphosphate (ATP) production and systemic lactic acidosis (Hanrahan et al., 1996; Shalak & Perlman, 2004; Wyatt et al., 1989). Subsequently, ATP-dependent Na+/K+ pump fails and results in neuronal depolarization, following which calcium influx occurs facilitated by activation of N-methyl-D-aspartate (NMDA) receptors and other excitotoxic neurotransmitters (Grow & Barks, 2002; Volpe J. J., 2008). Toxic cytoplasmic calcium concentrations then trigger various neurotoxic cascades, such as activation of nitric oxide synthase, mitochondrial dysfunction, production of reactive oxygen species (ROS), necrosis and/or apoptosis, and inflammatory cascades. (Allen & Brandon, 2011; Chakkarapani et al., 2021; Nair & Kumar, 2018)

Mast cells were demonstrated to be an early responder activated immediately after the initial hypoxic-ischemic (HI) insult and even before microglia activation in postnatal day 7 (P7) rats (Jin et al., 2009). Acute microglia activation was shown in white matter around 2 hours after HI insult in a P7 rat model (McRae et al., 1995). Necrosis, the major phenotype of cell death shown in various NE animal models including rats, mice and piglets (Northington et al., 2011), leads to cell rupture and cell content release, which can trigger activation and migration of microglia to the site of injury, resulting in additional inflammation (Allen & Brandon, 2011;

Alvarez-Díaz et al., 2007; Volpe J. J., 2008). It was demonstrated in the hypoxic neonatal rat models that the activated microglia vigorously produce excess amounts of inflammatory cytokines such as tumor necrosis factor α (TNFα) and interleukin (IL)-1β (Deng et al., 2008), together with glutamate (Sivakumar et al., 2010) and ROS (Kaur et al., 2013; Rathnasamy et al., 2011). Consequently, the developing blood-brain barrier (BBB) is disrupted and allows the infiltration of peripheral leukocytes, such as neutrophils and macrophages (Greco et al., 2020; Hagberg et al., 2015; Kaur et al., 2013; Liu & McCullough, 2013). In P7 rats, the expressions of pro-inflammatory cytokines IL-1β and TNFα significantly and transiently increased at 3 to 4 hours in the cortex and hippocampus following the initial HI insult (Hagberg et al., 1996; Szaflarski et al., 1995). This primary phase of injury happens within minutes to hours after the initial insult, and only very few therapeutic interventions have the ability to control this primary energy failure (Cardinali D. P., 2019; Nair & Kumar, 2018).

1.2.2 Latent phase

A latent period that follows the primary phase is thought to last 6 to 12 hours, and is associated with reduced cerebral metabolism and increased tissue oxygenation (Chakkarapani et al., 2021; Jensen et al., 2006). It opens up a "therapeutic window" for neuroprotective interventions (Nair & Kumar, 2018). However, the optimal timing and duration of this "therapeutic window" remains to be fully understood, thus limiting the efficacy of current available neuroprotective treatments (Chakkarapani et al., 2021; Wintermark, 2011).

1.2.3 Secondary phase

Secondary energy failure ensues, and typically starts around 6 to 8 hours after the HI insult and lasts for a few days (Chakkarapani et al., 2021; Davidson et al., 2021; Nair & Kumar, 2018). Although the exact mechanisms of secondary phase are again not completely understood similarly to the latent phase (Allen & Brandon, 2011; Cotten & Shankaran, 2010), they appear to be associated with mitochondrial dysfunction (Hagberg et al., 2014), oxidative stress, excitotoxicity, apoptosis and necrosis, and neuroinflammation (Chakkarapani et al., 2021; Nair & Kumar, 2018).

Recent evidences have suggested that there is a dynamic change of microglia phenotypes over the course of NE injury (Bonestroo et al., 2013; Davidson et al., 2021; Hellström Erkenstam et al., 2016). In P7 rats, an increase in the harmful pro-inflammatory phenotype of microglia was observed early at 3 hours after the HI insult and continued into the secondary phase until 24 hours after HI, while a "healing" phenotype only started to appear 24 hours after HI (Bonestroo et al., 2013; Davidson et al., 2021). In P7 mouse model of NE, both phenotypes were shown 6 hours after the insult and were progressively shifted to more harmful phenotype in the following 24 hours (Hellström Erkenstam et al., 2016). The harmful pro-inflammatory phenotype of microglia may contribute to the secondary injury phase of NE by their typical release of inflammatory mediators, such as TNFα, IL-1β, IL-6, monocyte chemoattractant protein-1 (MCP-1), and macrophage inflammatory protein-1 alpha (MIP-1α) (Chakkarapani et al., 2021). Astrocytes were found activated readily in all brain around 24 to 72 hours after HI using a P7 rat model (Bona et al., 1999). Reactive astrocytes release pro-inflammatory cytokines, such as IL-1, IL-6, TNF-α, and interferon-gamma (IFNγ), following ischemic events (Lau & Yu, 2001; Liu & McCullough, 2013). But also, astrocyte-produced inflammatory mediators IL-10 and IL-33 were

shown to confer neuroprotection in NE, by attenuating cell apoptosis in P7 rat and mouse models respectively (He et al., 2017; Jiao et al., 2020). Neutrophil infiltration to the infarct region was found around 12 hours following HI insult in P7 rats (Bona et al., 1999). Its participation in NE pathogenesis was also implied by the neuroprotective effect of neutrophil depletion induced before HI in P7 rats (Bona et al., 1999; Hudome et al., 1997; Palmer et al., 2004). Experimental studies have shown that in P7 rat brain tissue, IL-1β and TNFα significantly increased around 6 to 12 hours after HI (Bona et al., 1999); IL-1 and IL-6 significantly increased around 6 hours after HI (Hagberg et al., 1996); IL-10 significantly increased from 6 to 48 hours after HI (Li et al., 2014); and IL-18 gradually increased over the course of injury starting around 24 hours after HI (Hedtjärn et al., 2002). Intriguingly, a study in adult HI mice indicated that IL-1β may mediate the chemotaxis of peripheral immune cells to injury site through induction of β-chemokines MIP-1α, MIP-1β, MCP, and regulated on activation normal T cell expressed and secreted (RANTES) (Lazovic et al., 2005; Leonardo & Pennypacker, 2009). As studied in a P7 rat model of NE, α-chemokine MIP-2 was found elevated from 0 to 12 hours after HI accompanied by the increase in IL-1β and TNFα; β-chemokines MIP-1α was induced around 6 hours to 14 days after HI, MIP-1β was induced approximately 1 to 6 hours after HI, and RANTES was induced around 24 hours to 14 days after HI (Bona et al., 1999).

1.2.4 Tertiary phase

In recent years, researchers have started to recognize the presence of a tertiary phase of NE that might persist for months to years following the initial injury, where continuous active mechanisms may prevent neuronal regeneration, exacerbate brain damage, and sensitise the brain to further injury (Dammann, 2007; Davidson et al, 2021; Fleiss & Gressens, 2012). These

mechanisms of damage primarily include persistent inflammation and altered epigenome, thus creating a toxic niche hindering normal neurodevelopmental processes, such as neurogenesis, synaptogenesis, oligodendrocyte maturation, and axonal growth (Favrais et al., 2011; Fleiss & Gressens, 2012; Leviton & Gressens, 2007). Multiple immune cells were found activated in the tertiary stage. Lymphocytes demonstrated minimal involvement in the early phases of NE, but started invading the infarct region later around 7 days after HI in P7 rats (Bona et al., 1999; Liu & McCullough, 2013). It was found that the activation of microglia/macrophages, astrocytes, and CD4 lymphocytes persisted up to at least 35 days after HI in P7 rats, suggesting a chronic inflammatory state in NE (Bona et al., 1999). This tertiary phase potentially provides a prolonged therapeutic window for neurorestorative interventions that target those mechanisms involved (Chakkarapani et al., 2021; Davidson et al, 2021: Fleiss & Gressens, 2012).

1.3 Clinical implication of inflammatory biomarkers in neonatal encephalopathy (NE)

The important role that inflammation plays throughout the pathophysiological stages of NE has long been recognized, but is not fully defined (Ahearne et al., 2017; Chakkarapani et al., 2021; Fleiss & Gressens, 2012; Nair & Kumar, 2018). It is not surprising that inflammatory markers have been extensively studied as predictive biomarkers for NE diagnosis, severity, and/or outcomes.

Prior clinical studies have identified several inflammatory biomarkers that were elevated or decreased following HI insult, and were associated with worse injury and/or neurological outcome in neonates with NE (**Table 1**). Increased IL-6 and IL-16 levels in umbilical cord plasma at birth demonstrated strong association with higher severity of NE in 69 term neonates with NE treated with therapeutic hypothermia (TH) (Walsh et al., 2013). Thirty-three term

neonates with NE from the same cohort were assessed later at 3 years of age for neurodevelopmental outcome, and the elevated IL-16 was found predictive of severely abnormal neurological outcome (Ahearne et al., 2017). Higher neonatal blood levels of IL-1β, IL-6, IL-8, and TNFα were associated with elevated lactate/choline ratio measured by magnetic resonance spectroscopy (MRS), indicating impaired cerebral oxidative metabolism on 2-16 days of life in 42 term or near-term neonates at risk of NE. In the same study, increased IL-1β, IL-6 and IL-8, and decreased IL-12 were found associated with death, or cognitive delay and/or functional motor deficit detected at 30 months of age in 54 term or near-term neonates at risk of NE (Bartha et al., 2004). In a prospective cohort study that involved 103 neonates diagnosed with NE and treated with TH, elevated plasma levels of IL-6, IL-8, and IL-10 within 24 hours of life, and Tau at 72 to 96 hours of life, accurately predicted severe brain injury seen on magnetic resonance imaging (MRI). The late Tau measurement also strongly associated with death or significant neurodevelopmental delay at over 1 year of age (McGowan et al., 2021). Elevated glial fibrillary acidic protein (GFAP), IL-1, IL-6, IL-8, TNF, IFN, and vascular endothelial growth factor (VEGF) in arterial blood collected at 6 to 24 hours of life were found predictive of abnormal neurological outcome at 15 to 18 months of age in 20 neonates with moderate to severe NE treated with TH (Chalak et al., 2014). Not only blood biomarkers, but also inflammatory markers from cerebrospinal fluid (CSF) have been studied in human neonates with NE. Increased level of IL-6 in CSF collected within 48 hours of life from 90 neonates diagnosed with NE was found related with severe brain injury but not mild or moderate, and with adverse neurological outcome at 12 months of age. In the meanwhile, the level of VEGF in CSF collected 72 hours after birth from the same group of neonates was shown to be associated with the degree of NE (Vasiljevic et al., 2011). Another study in CSF inflammatory biomarkers involved 20 neonates with NE, and

discovered that the levels of both IL-6 and IL-8 within 72 hours of life were associated with the severity of NE. In addition, elevated IL-6 in CSF was confirmed again to be linked with abnormal outcomes classified by disabling abnormalities in tone or reflexes, seizures, or blindness (Sävman et al., 1998).

These studies suggested that understanding of the link between inflammatory biomarkers and NE injury and/or outcome may aid in prognostication in neonates with NE. Inflammatory biomarkers may serve as guidance for therapeutic interventions (Orrock et al., 2016) and may be used to assess the impact of therapeutic approaches (Jenkins et al., 2012; Massaro et al., 2018).

1.4 Therapeutic hypothermia (TH)

To date, the only recognized standard care for moderate to severe NE is therapeutic hypothermia (TH) that includes cooling the whole body to a temperature of 33.5 ± 0.5°C initiated within 6 hours of birth and continued for 72 hours, followed by a slow rewarming at 0.5°C per hour up to normothermia (Shankaran, 2012; Wintermark, 2011). TH exerts its neuroprotective effects mainly by reducing brain perfusion and metabolism, with parallel decrease in oxygen demand, reducing free radical production, alleviating reperfusion injury, suppressing apoptosis and epileptic activity, as well as modulating inflammation (Drury et al., 2010; Polderman, 2008; Wintermark, 2011). In experimental study, TH was shown to suppress activated microglia in injured white and grey matter in a preterm hypoxic fetal sheep model (Bennet et al., 2007). In clinical investigations, TH was demonstrated to have effects on blood inflammatory biomarkers that were previously found indicative of the severity and/or neurological outcome of NE. TH appeared to be associated with lower serum level of pro-inflammatory cytokine IL-6 and higher serum level of anti-inflammatory cytokine IL-10 at 48

hours after birth in 12 neonates with NE treated with TH comparing to 8 normothermic neonates with NE (Moon et al., 2016). By comparing 28 TH-treated neonates with NE with 22 normothermic neonates with NE, TH upregulated the serum levels of MCP-1, IL-6, IL-8 and IL-10 over the first 80 hours of life (Jenkins et al., 2012). In the same cohort of TH-treated neonates, those who had better neurodevelopmental outcomes at 12 months of age exhibited a generally lower serum levels of IL-6, IL-8 and IL-10 from 24 to 36 hours after starting treatment, suggesting that TH may confer neuroprotection in partial neonates with NE by modulating inflammation (Jenkins et al., 2012).

With only little adverse effects, TH appears to reduce mortality and developmental disability at 18 months of age (Edwards et al., 2010; Jacobs et al., 2013), and this improvement continues into childhood (Shankaran, 2012). However, TH solely attempts to prevent the development of brain injury by targeting the latent and secondary phases of NE, but fails to repair the injuries once they developed (Chakkarapani et al., 2021; Gluckman et al., 2005; Shankaran et al., 2005; Yazdani et al., 2016). Moreover, TH does not confer neuroprotection in all cases. It seems mostly effective in neonates with moderate NE, but not in many neonates with severe NE (Azzopardi et al., 2009; Gluckman et al., 2005). Although TH was shown to modulate inflammatory responses to HI insult and result in better neurological outcome in partial experimental and clinical NE subjects without infection/inflammation (Bennet et al., 2007; Drury et al., 2010; Jenkins et al., 2012; Moon et al., 2016; Polderman, 2008), evidences have suggested that its effectiveness in neuroprotection is limited when pre-existing infection/inflammation is present concomitantly. The protection by TH was significantly reduced in a P7 rat model primed with infection prior to HI insult (Osredkar et al., 2014). In a study with 23 neonates with NE, TH was shown to be less effective in reducing brain injury when chorioamnionitis, an intrauterine

infection/inflammation, was detected (Wintermark et al., 2010). In addition, sepsis as a result of infection has been considered as one of the major contributing factors that limit TH effectiveness in low-and-middle-income countries (Nair & Kumar, 2018; Pauliah et al., 2013). On top of that, the timing of initiation and duration of TH largely affect its effectiveness (Davidson et al., 2018; Gunn, A. J., & Gunn, T. R., 1998). Therefore, it is in urgent need to find adjunctive treatments that are simple and safe to complement TH and improve the outcomes in neonates with moderate to severe NE.

1.5 Adjunctive treatments

Modulating neuroinflammation appears thus a reasonable therapeutic target for new adjunctive treatments (Ahearne et al., 2017; Chakkarapani et al., 2021; Davidson et al., 2021; Drury et al., 2010; Fleiss & Gressens, 2012; Jenkins et al., 2012; Moon et al., 2016; Nair & Kumar, 2018; Polderman, 2008; Wintermark, 2011). It may also help improve the limited effectiveness of TH when prior infection/inflammation exists (Nair & Kumar, 2018; Osredkar et al., 2014; Pauliah et al., 2013; Wintermark et al., 2010). Furthermore, continuing to mediate inflammation in the tertiary phase of NE may help create a healthier neuronal environment to support the restoration of neurodevelopmental processes, and thus provide a neurorestorative effect that TH lacks (Favrais et al., 2011; Fleiss & Gressens, 2012; Leviton & Gressens, 2007).

1.6 Sildenafil

Sildenafil is a promising candidate for adjunctive therapy. It is a highly selective inhibitor of phosphodiesterase type-5 (PDE5) and a potent vasodilator that can cross the blood-brain barrier (Chakkarapani et al., 2021; Ghofrani et al., 2006; Peixoto et al., 2015). Sildenafil

increases the intracellular level of cyclic guanosine monophosphate (cGMP) by inhibiting its breakdown by PDE5 (Ghofrani et al., 2006). The accumulation of cGMP activates the downstream effectors via phosphokinase G (PKG), and the cGMP-PKG pathway has been evidenced to regulate neurogenesis, myelination, synaptic plasticity, cognition, and neuroinflammation in adult brains (Chakkarapani et al., 2021; Peixoto et al., 2015). In lipopolysaccharide (LPS)-stimulated rat primary astrocytes, the activation of the cGMP-PKG pathway reduced the matrix metalloproteinase-9 (MMP-9) (Shin et al., 2007), which has been shown as elevated in adult neurological diseases including multiple sclerosis and ischemia (Yong et al., 2001). In addition, cGMP was involved in diminishing TNFα-induced expression of MCP-1 in human umbilical vein endothelial cells, suggesting its role in modulating inflammatory responses (Weber et al., 2003). Moreover, PDE inhibitors have been shown to mitigate microglia activation and production of TNFα in adult rat models of cerebral hypoperfusion (Wakita et al., 2003) and multiple sclerosis (Fujimoto et al., 1999). Therefore, PDE5 inhibitors such as sildenafil are promising therapeutic strategies for neurological diseases (Chakkarapani et al., 2021; Peixoto et al., 2015).

1.6.1 Neurorestorative effects of sildenafil on adult stroke

Sildenafil has been shown to exert neurorestorative effects in adult stroke. It was found to promote neurogenesis, synaptogenesis, angiogenesis, axonal remodeling, and functional recovery in rat models of adult stroke (Bednar, 2008; Ding et al., 2008; Li et al., 2007; Zhang et al., 2002). Furthermore, sildenafil has been tested in human patients with stroke. It was shown to be safe in young to aged adult patients with mild to moderately severe stroke (Silver et al., 2009). While sildenafil has been intensively studied in adult stroke, little investigations were done in the

context of NE. The immature neonatal brain is still under development at the time of birth, and thus cannot be simply considered as a smaller version of the adult brain. The mechanisms underlying NE is most likely very distinct from those underlying adult stroke (Jensen, 2006; Wintermark, 2011). It is critical for sildenafil to be investigated in neonates with NE.

1.6.2 Inflammation-modulating potentials of sildenafil

Sildenafil has demonstrated anti-inflammatory effects in many neurological situations. An in vitro study has demonstrated that sildenafil exerted anti-inflammatory effects on LPS-induced microglial cells obtained from mouse embryonic cultures, by blocking nuclear factor-κB (NFκB) and mitogen-activated protein kinase (MAPK) activation, and as a result significantly suppressed the production of IL-1β and TNFα (Zhao et al., 2011). Mounting evidences also reported that sildenafil can reduce neuroinflammation in adult neurological conditions, such as multiple sclerosis (MS) (Araújo et al., 2020; Nunes et al., 2015) and Alzheimer's disease (AD) (Zhang et al., 2013). In the hippocampus of an adult mouse model of MS, sildenafil successfully reduced the infiltration of CD4 lymphocytes and the IL-17 and TNFα they produced; down-modulated pro-inflammatory proteins, including IL-1β, phospho-IκBα and phospho-NFκB; and upregulated anti-inflammatory cytokines transforming growth factor-beta (TGFβ) and IL-10 (Araújo et al., 2020). Another study in MS adult mouse model demonstrated that sildenafil can attenuate reactive astrogliosis and microglia activation in cerebellum, with parallel decrease in NFκB and increase in IL-10, and reduce the serum level of cytokines TNFα and IL-1β (Nunes et al., 2015). Sildenafil was reported to significantly inhibit the increase of pro-inflammatory cytokines TNFα, IL-1β, and IL-6 induced by β-amyloid (Aβ) in the hippocampus of a mouse model of AD, and this effect may have acted via the cGMP-PKG pathway (Zhang et al., 2013).

1.6.3 Neurorestorative effects of sildenafil on animal model of NE

Intriguingly, sildenafil is already used as a safe treatment in neonates with pulmonary hypertension (Simonca & Tulloh, 2017), but only few systematic studies have investigated its effects on the neonatal brain. In rat models of NE, sildenafil was indicated to mediate cerebral blood flow (CBF) redistribution, mitigate HI damage, promote neurogenesis, improve motor locomotion, and reduce apoptosis and inflammation (Charriaut-Marlangue et al., 2014; Yazdani et al., 2016, Yazdani et al., 2021). An intraperitoneal injection of sildenafil immediately after HI insult in P7 rats inhibited reactive astrogliosis and microglia activation at 72 hours and 7 days post-HI, suggesting the anti-inflammatory ability of sildenafil in NE (Charriaut-Marlangue et al., 2014). Our preliminary results in rat model of NE also showed that sildenafil treatment reduces neuroinflammation by decreasing the number of microglia and astrocytes, and by modulating expression of the cytokines TNFα, IL-1β, and IL-1 receptor antagonist (IL-1Ra) (Yazdani et al., 2021).

1.7 Translation into clinical trials in human neonates

Animal models of NE cannot fully reproduce the human condition (Bracken, 2009), especially when NE has many complex contributing factors (Aslam et al., 2019; Gillam-Krakauer & Gowen, 2021). It is very important to further explore the use of sildenafil in human neonates with NE. The translation into clinical trial is greatly supported by the previously described benefits of sildenafil in the rat models of NE (Charriaut-Marlangue et al., 2014; Yazdani et al., 2016; Yazdani et al., 2021), and the consistent safety profile of sildenafil in

animal models, adult patients with stroke (Silver et al., 2009), and human neonates with persistent pulmonary hypertension (Simonca & Tulloh, 2017).

1.8 Rationale for the thesis study, hypothesis, and specific aims

The limitations of TH, the only current treatment for NE, raise the urgent need for adjunctive treatments to complement TH by protecting and/or repairing brain injuries in neonates with NE. Modulating inflammation appears to be a promising approach, since neuroinflammation is an important pathophysiological mechanism of NE (Ahearne et al., 2017; Chakkarapani et al., 2021; Fleiss & Gressens, 2012; Nair & Kumar, 2018), and mitigating it appears to decrease adverse outcome (Jenkins et al., 2012). In addition, reducing inflammation may provide neurorestoration by creating a less toxic environment for neurodevelopmental processes in the tertiary phase of NE (Favrais et al., 2011; Fleiss & Gressens, 2012; Leviton & Gressens, 2007). Sildenafil has been found to have inflammation-modulating effects by reducing astrocyte and microglia activation and by modulating inflammatory cytokines in animal models of various adult neurological diseases and in rat models of NE (Araújo et al., 2020; Charriaut-Marlangue et al., 2014; Nunes et al., 2015; Yazdani et al., 2021; Zhang et al., 2013). The impact of sildenafil on inflammation in human neonates with NE remains to be determined.

Our objective is to investigate blood inflammatory biomarkers that are associated with brain injury in neonates with NE treated with TH, and study if sildenafil has an impact on them. We hypothesize that inflammatory biomarkers may provide early guidance for therapeutic interventions in the context of NE, and sildenafil may keep its inflammation-modulating effects in human neonates with NE.

<u>Aim 1</u>: Explore inflammatory biomarkers that are associated with brain injury in neonates with NE (Chapter 2). We analyzed inflammation-related protein levels in plasma samples collected on day 2 of life from 2 groups of neonates: near-term and term neonates with NE (1) without brain injury treated only with TH, and (2) with brain injury treated only with TH.

<u>Aim 2</u>: Evaluate the impact of sildenafil on inflammatory markers in early days of NE (Chapter 3). We analyzed the inflammation-related protein levels in plasma samples collected on day 2, 3, and 4 of life from 3 groups of neonates: near-term and term neonates with NE (1) without brain injury treated only with TH, (2) with brain injury treated only with TH, and (3) with brain injury treated with TH and sildenafil.

1.9 Tables

Table 1. Clinical implication of inflammatory biomarkers in neonatal encephalopathy (NE)

Newborns investigated	Therapeutic hypothermia	Biomarker	Analyte level	Sample source	Time point	Clinical implication	Reference
69 term asphyxiated newborns, and 61 control newborns	Received	**IL-6, IL-16**	Elevated	Umbilical cord plasma	At birth	Associated with higher severity of NE determined by continuous EEG monitor and Sarnat score[1] at 24 hours of life	Walsh et al., 2013
33 term asphyxiated newborns	Received	**IL-16**	Elevated	Umbilical cord plasma	At birth	Associated with severely abnormal neurodevelopmental outcome at 3 years of age defined by BSID-III score[2]	Ahearne et al., 2017
42 term or near-term newborns at risk of NE	Information not available	**IL-1β, IL-6, IL-8, TNFα**	Elevated	Capillary blood	On the first days of life	Associated with impaired cerebral oxidative metabolism measured by MRS on 2-16 days of life	Bartha et al., 2004
54 term or near-term newborns at risk of NE	Information not available	**IL-1β, IL-6, IL-8** **IL-12**	Elevated Decreased	Capillary blood	On the first days of life	Associated with death, or cognitive delay and/or functional motor deficit detected at 30 months of age	Bartha et al., 2004
103 newborns with NE	Received	**IL-6, IL-8, IL-10**	Elevated	Plasma	Within 24 hours of life	Associated with severe brain injury seen on MRI on 4-7 days of life	
		Tau	Elevated	Plasma	72-96 hours of life	Associated with death, or severe brain injury seen on MRI on 4-7 days of life, and/or significant neurodevelopmental delay defined by BSID-III score[2] over 1 year of age	McGowan et al., 2021
20 newborns with moderate to severe NE	Received	**GFAP, IL-1, IL-6, IL-8, VEGF, TNF, IFN**	Elevated	Arterial blood	6-24 hours of life	Associated with abnormal neurological outcome at 15-18 months of age defined by BSID-III score[2]	Chalak et al., 2014
		IL-6	Elevated	CSF	Within 48 hours of life	Associated with higher severity of NE determined by Sarnat score[1] and aEEG, and adverse neurological outcome at 12 mongths of age assessed using Denver Developmental Screening Test[3]	
90 newborns with NE	Information not available	**VEGF**	Elevated	CSF	72 hours after birth	Associated with degree of NE determined by Sarnat score[1] and aEEG	Vasiljevic et al., 2011
		IL-6, IL-8	Elevated	CSF	Within 72 hours of life	Associated with degree of NE determined by Sarnat score[1]	
20 asphyxiated newborns, and 7 control newborns	Information not available	**IL-6**	Elevated	CSF	Within 72 hours of life	Associated with abnormal outcomes classified as disabling abnormalities in tone or reflexes, seizures, or blindness	Sävman et al., 1998

[1]Sarnat score (Sarnat & Sarnat, 1976)

[2]Bayley Scales of Infant and Toddler Development (Edition III): indicative of abnormal neurological outcome if score < 85 (Bayley, 2006).

[3]Denver Developmental Screening Test (Frankenburg & Dodds, 1967)

Chapter 2

Plasma inflammatory biomarkers associated with brain injury in neonates with neonatal encephalopathy treated with therapeutic hypothermia

Ruofan Song, Emmanouil Rampakakis, Pia Wintermark

Status of the Article: In preparation for submission to a journal

Foreword: The expression of inflammatory biomarkers remains unclear in the neonates with NE who still develop brain injury despite the treatment of TH. In this Chapter 2, we explored plasma inflammatory biomarkers that were associated with brain injury seen on day-2 brain MRI in neonates with NE treated with TH. Inflammatory protein levels in plasma were quantified using multi-analyte profiling antigen analysis. An upregulation of eotaxin-1, interleukin (IL)-12p40, pulmonary and activation-regulated chemokine (PARC), and stem cell factor (SCF) was observed in neonates with brain injury, compared to those without. Our findings suggest a potential involvement of these cytokines (IL-12p40 and SCF) and chemokines (eotaxin-1 and PARC) in the development of brain injury in neonates with NE treated with TH. This study enhanced our understanding of the link between inflammatory biomarkers and brain injury in NE. These inflammatory biomarkers may represent novel therapeutic targets, and may serve as indicators for the impact of new therapeutic approaches on brain injury in NE.

2.1 Abstract

Background: Many neonates with neonatal encephalopathy (NE) still develop brain injury despite therapeutic hypothermia (TH) treatment. Inflammation-related proteins appear to be involved in the pathophysiology of NE, yet their profile in the neonate with NE who do not respond to TH adequately remains unclear.

Objective: To investigate the plasma inflammatory biomarkers that are associated with the brain injury in neonates with NE treated with TH.

Methods: Plasma samples were collected on day 2 of life (DOL 2) in term and near-term neonates with NE treated with TH with and without brain injury seen on magnetic resonance imaging (MRI). Twenty-five inflammatory markers in these samples were quantified by multi-analyte profiling antigen analysis, and compared between the neonates with and without brain injury using Mann-Whitney U tests.

Results: Thirty-five neonates with NE treated with TH were included in this study, including 5 without brain injury and 30 with brain injury. On day 2 of life, the plasma levels of cytokines interleukin (IL)-12p40 and stem cell factor (SCF), and chemokines eotaxin-1 and pulmonary and activation-regulated chemokine (PARC), were significantly elevated in the neonates with NE treated with TH with brain injury, compared to those without.

Conclusion: Inflammatory proteins IL-12p40, SCF, eotaxin-1 and PARC might be associated with the development of brain injury in neonates with NE despite TH treatment.

2.2 Introduction

Neonatal encephalopathy (NE) as a result of birth asphyxia (Aslam et al., 2019; Hassell et al., 2015; Shalak & Perlman, 2004; Volpe J. J., 2012) accounts for an estimated 23% of neonatal mortality worldwide (Bryce et al., 2005), and burdens surviving children with adverse long-term morbidities, such as cerebral palsy, motor impairments, intellectual disabilities, and epilepsy (Al-Macki et al., 2009; Wintermark, 2011). Currently, the only standard care for moderate to severe NE is therapeutic hypothermia (TH) (Shankaran, 2012; Wintermark, 2011), yet many treated neonates still develop brain injury and bear significant neurological disabilities in long-term (Azzopardi et al., 2009; Cheong et al., 2012; Gluckman et al., 2005; Wintermark, 2011). Adjunctive treatments that can complement TH are in urgent demand, and so are clinical biomarkers that can identify neonates in need of adjuvant therapies (Chalak L. F., 2016; McGowan et al., 2021; Orrock et al., 2016).

Neuroinflammation has long been recognized to play a critical role throughout the pathophysiological stages of NE (Ahearne et al., 2017; Chakkarapani et al., 2021; Fleiss & Gressens, 2012; Nair & Kumar, 2018), and thus appears an interesting target for new adjunctive treatments. Some inflammation-related proteins, such as interferon (IFN), interleukin-1 beta (IL-1β), IL-6, IL-10, and tumor necrosis factor alpha (TNFα), have been shown to participate in the neuroinflammatory cascades activated by hypoxic-ischemic (HI) insult in rodent models of NE (Bona et al., 1999; Deng et al., 2008; Hagberg et al., 1996; He et al., 2017; Jiao et al., 2020; Lau & Yu, 2001; Li et al., 2014; Liu & McCullough, 2013; Szaflarski et al., 1995), and have also been widely studied as biofluid markers predictive of severity and/or outcome in human neonates with NE (Bartha et al., 2004; Chalak et al., 2014; McGowan et al., 2021; Sävman et al., 1998; Walsh et al., 2013; Vasiljevic et al., 2011). However, investigations of inflammatory biomarkers

in neonates with NE that have been treated with TH have just started in recent years (McGowan et al., 2021). The knowledge is limited pertaining to the profile of inflammatory proteins that play a role in the neonates with NE who do not respond well to TH (Orrock et al., 2016; Walsh et al., 2013).

In this study, we aimed to explore the plasma inflammatory biomarkers that were associated with brain injury in neonates with NE despite TH treatment, in the hope to use them as possible biomarkers to select neonates in need of additional interventions than TH.

2.3 Methods

2.3.1 Patients

Male and female near-term and term neonates with moderate and severe NE and treated with hypothermia were prospectively enrolled in this cohort study. Criteria for TH were those previously described (Azzopardi et al., 2009; Gluckman et al., 2005; Shankaran et al., 2005): (1) gestational age ≥ 36 weeks and birth weight ≥ 1800 grams; (2) evidence of fetal distress, i.e., history of an acute perinatal event, any occurrence of cord pH ≤ 7.0 or base deficit ≤ -16mEq/L; (3) evidence of neonatal distress, such as an Apgar score ≤ 5 at 10 minutes after birth, postnatal blood gas pH obtained within the first hour of life ≤ 7.0 or base deficit ≤ -16mEq/L, or a need for ventilation initiated at birth and continued for at least 10 minutes; and (4) moderate to severe NE evidenced by an abnormal neurological exam and/or an aEEG. Eligible neonates received whole-body cooling to an esophageal temperature of 33.5°C, initiated within the first 6 hours of life, continued for 72 hours, and followed by slowly rewarming.

A subset of these neonates demonstrated brain injury on their day-2 brain magnetic resonance imaging (MRI) (Boudes et al., 2015; Wintermark et al., 2011; Wisnowski et al., 2021).

The MRI results were reviewed and scored by neuroradiologists blinded to neonates' clinical conditions according to a previously described MRI scoring system (Barkovich et al., 1998).

Clinical characteristics for each neonate were collected, including gestational age, birth weight, gender, Apgar score at 10 minutes, arterial cord pH, initial postnatal blood gas pH, initial modified Sarnat score on admission, initial aEEG background activity, outcome (i.e., alive or dead), white blood cell (WBC) counts on day 2 of life, and placental pathology results.

The research protocol was approved by the research ethics board from the Montreal Children's Hospital, McGill University Health Centre, and informed written consent was obtained from the parents on behalf of their neonate prior to any study-specific procedures.

2.3.2 Inflammation-related proteins

Blood samples were collected into potassium EDTA coated microtainers on day 2 of life for enrolled neonates. All the samples were immediately centrifuged after collection at 3600 rpm for 6 minutes at room temperature. Afterwards, plasma was removed, aliquoted, coded and stored at -80°C until tested.

Protein markers were measured blinded to patient identity and disease. The samples were thawed at room temperature, vortexed, spun at 13,000 x g for 5 minutes for clarification before 40 mcL were transferred to a master microtiter plate for multi-analyte profiling (MAP) antigen analysis. Using automated pipetting, an aliquot of each sample was introduced into one of the capture microsphere multiplexes of the Rules-Based Medicine (Myriad RBM) Custom Human multi-analyte profile (Myriad, Austin, TX) (http://www.rules-basedmedicine.com). These sample and capture microspheres mixtures were fully mixed and incubated at room temperature for 1 hour. Subsequently, multiplexed cocktails of biotinylated reporter antibodies were added into

each multiplex robotically and, after thoroughly mixed, were incubated for another 1 hour at room temperature. An excess of streptavidin-phycoerythrin solution was thoroughly mixed into each multiplex and incubated for 1 hour at room temperature to develop the multiplexes. The volume of each multiplexed reaction was reduced by vacuum filtration and the volume increased by dilution into matrix buffer for analysis. The multiplexes were analyzed with a Luminex 100 instrument and the resulting data stream was interpreted using proprietary data analysis software developed at Rules-Based Medicine (Myriad RBM). For each multiplex, both calibrators and controls were included on each microtiter plate. The first and last columns of each plate were used to run 8-point calibrator, and 3-level controls were included in duplicate. To ensure proper assay performance, testing results were determined first for the high, medium and low controls for each multiplex. Unknown values for each analyte that localized in a specific multiplex were obtained using 4- and 5- parameter weighted and non-weighted curve fitting algorithms included in the data analysis package. Expression of 25 inflammation-related proteins was analyzed. Analyzed proteins, listed in **Table 1**, were selected based on inflammatory involvement and assay availability. Plasma concentrations were reported for each sample, unless the sample quantity was not sufficient (QNS), the concentration was below the lower limit of quantification (LLOQ), or the concentration was above the highest quantifiable value.

2.3.3 Data analysis

Descriptive data including the mean and standard deviation for continuous variables and the frequency distribution for categorical variables were generated.

Mann-Whitney U tests for continuous variables and Chi-square tests for categorical variables were conducted to compare the differences of clinical characteristics between neonates

with and without brain injury. Mann-Whitney U tests were used to assess for statistical significance of the differences in inflammatory protein expressions between the two groups of neonates.

A p value < 0.05 was considered as statistically significant. All statistical analyses were performed with SPSS Version 28.0 for Mac (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA).

2.4 Results

2.4.1 Patients

Thirty-five neonates with NE treated with TH were included in this study. Five neonates with NE did not develop brain injury, while the remaining 30 neonates developed brain injury. No significant difference in the clinical characteristics was observed between the two groups (**Table 2**).

2.4.2 Expression of plasma inflammatory biomarkers was different on day 2 of life between neonates without and with brain injury

The plasma concentrations of three pro-inflammatory cytokines IL-1α, IL-1β, and IL-12p70 were below the LLOQ in all (5/5) neonates with NE without brain injury. In addition, IL-1α had plasma concentrations below LLOQ in 96% (29/30) of the neonates with brain injury; IL-1β had plasma concentrations below LLOQ in 86% (26/30) of these neonates; and IL-12p70 had plasma concentrations below LLOQ in 80% (24/30) of these neonates. Therefore, these three cytokines were excluded from further statistical analysis.

Four inflammation-related proteins were found significantly different on day 2 of life between neonates with NE treated with TH without and with brain injury (**Fig. 1**). The plasma concentrations of cytokine IL-12p40 in neonates with brain injury (0.38 ± 0.24 ng/mL) were significantly higher than in neonates without brain injury (0.19 ± 0.05 ng/mL) ($p = 0.04$). Plasma stem cell factor (SCF) was also significantly elevated in neonates with brain injury (558.86 ± 331.41 pg/mL), compared to neonates without brain injury (324.80 ± 78.99 pg/mL) ($p = 0.04$). The chemokine eotaxin-1 had significantly higher plasma concentrations in neonates with brain injury (164.00 ± 59.32 pg/mL), compared to neonates without brain injury (92.00 ± 12.19 pg/mL) ($p = 0.04$). The plasma level of pulmonary-and activation-regulated chemokine (PARC) was also significantly increased in neonates with brain injury (39.83 ± 22.43 ng/mL) than in neonates without brain injury (17.10 ± 13.83 ng/mL) ($p = 0.03$).

Other tested inflammatory markers had no significant difference between the 2 groups.

2.5 Discussion

In our study, there was a significant upregulation on day 2 of life of the cytokines IL-12p40 and SCF, and the chemokines eotaxin-1 and PARC, in the neonates with NE treated with TH with brain injury compared to those without (**Fig. 1**), corroborating the idea that inflammatory cascades are activated during brain injury development in the context of NE (Ahearne et al., 2017; Chakkarapani et al., 2021; Fleiss & Gressens, 2012; Nair & Kumar, 2018).

IL-12p40 is a subunit of the pro-inflammatory cytokine IL-12 and at the same time may function as an individual cytokine (Cooper & Khader, 2007; Gee et al., 2009; Trinchieri, G., 2003). Activated microglia were shown to produce IL-12 and IL-12p40 (Aloisi et al., 1997), and the IL-12 signaling in macrophages appeared to be a major pathway activated in human neonates

with NE within 12 hours of birth (Montaldo et al., 2019). One prior study that involved 13 neonates with NE treated with TH and 39 healthy control neonates demonstrated significantly increased serum level of IL-12p40 at birth in those with NE, compared to healthy controls (Go et al., 2021). But no significant difference of the IL-12p40 serum level at birth was observed between 5 neonates with NE treated with TH who had cerebral palsy (CP) and 8 without CP (Go et al., 2021). These findings suggested the involvement of IL-12p40 in NE injury but not specifically in neonates who still developed CP despite the treatment of TH. In a pre-clinical study using nonhuman primate model of NE, the plasma level of IL-12p40 was significantly lower at 24 and 72 hours of life in the 14 animals died or survived with CP, compared to the 8 animals survived without CP (Wood et al., 2021). This study indicated a different expression level of IL-12p40 in NE injury development, however, at a different timing than in the clinical study mentioned above.

Interestingly, IL-12p40 has been recognized to display both anti- and pro-inflammatory properties. It exhibited antagonizing effects specifically against IL-12 in mouse splenocytes, mainly by inhibiting the synthesis of IFNγ activated by IL-12 (Mattner et al., 1993). The murine homodimer IL-12p40 also appears a strong antagonist against IL-12 by competitively binding to their common receptor IL-12Rβ1 (Cooper & Khader, 2007; Gately et al., 1996; Gillessen et al., 1995; Vignali & Kuchroo, 2012). On the other hand, IL-12p40 was found associated with the pathological macrophages accumulation in human and mouse airway epithelial cells (Walter et al., 2001), and the IL-12p40 homodimer selectively chemoattracted macrophages via IL-12Rβ1 (Ha et al., 1999; Russell et al., 2003). Intriguingly, IL-12Rβ1 appears a key component for both anti- and pro-inflammatory signallings of IL-12p40 (Cooper & Khader, 2007; Gately et al., 1996; Gillessen et al., 1995; Ha et al., 1999; Russell et al., 2003; Vignali & Kuchroo, 2012). In light of

the aforementioned contradicting findings on IL-12p40 in NE, further elucidation is needed on whether the elevated IL-12p40 in our finding is activated by NE injury to counterregulate inflammatory activities, or is contributing to NE injury by promoting immune cells migration. IL-12Rβ1 may be an interesting therapeutic target for regulating the effects of IL-12p40.

SCF is an important growth factor, activator, and chemoattractant for mast cells (Nilsson et al., 1994; Reber et a., 2006), which were shown to be early responders activated immediately after the initial HI insult in postnatal day 7 (P7) rat model of NE (Jin et al., 2009). The production of SCF is upregulated under inflammatory conditions by pro-inflammatory cytokines such as IL-18, IL-1β, and TNFα (Reber et a., 2006), of which the levels have been shown to be increased following NE injury (Bona et al., 1999; Hagberg et al., 1996; Hedtjärn et al., 2002; Sivakumar et al., 2010; Szaflarski et al., 1995). These together suggested a potential association between the elevated level of SCF and the NE injury evolvement, which may explain the increased SCF in neonates who developed brain injury in our study. As far as we know, the investigation on SCF alone specifically in neonatal brain injury is very limited, and the relevant studies were mostly focusing on its synergistic effects with granulocyte-colony stimulating factor (G-CSF) (Doycheva et al., 2013; Dumbuya et al., 2021; Keller et al., 2006; Neubauer et al., 2016; Posod et al., 2019). In a neonatal mouse model of N-methyl-D-aspartate (NMDA) receptor-mediated developmental excitotoxic brain damage, administration of G-CSF+SCF resulted in deleterious enhancement of cortical and white matter lesions (Keller et al., 2006). Although not reflecting the independent effects of SCF itself, this discovery is consistent with our finding that SCF may participate in brain injury development. Opposite opinions also exist: injections of G-CSF+SCF displayed neuroprotective effects in rat NE model and neonatal mouse excitotoxic brain injury model, by preventing brain atrophy, reducing apoptotic cell deaths, and improving

neurological outcome (Doycheva et al., 2013; Neubauer et al., 2016). These improvements may have benefited from the neurorestorative (i.e., neurogenesis and angiogenesis) effects of SCF which have been revealed in adult brain injury models (Keller et al., 2006; Zhao et al., 2013).

Eotaxin-1, also known as C-C motif chemokine ligand 11 (CCL11), is a chemokine produced by a variety of cell types, including choroid plexus epithelial cells, astrocytes, and microglia, in the central nervous system (CNS) upon inflammatory stimulation (Baruch et al., 2013; Teixeira et al., 2018). It has been a recognized chemoattractant for leukocytes such as eosinophil, macrophages, and microglia, as well as neural progenitor cells (NPCs) and epithelial cells (Teixeira et al., 2018; Wakabayashi et al., 2021). Not only promoting the recruitment of microglia, eotaxin-1 was demonstrated to enhance the subsequent production of reactive oxygen species (ROS), and the glutamate-mediated neuronal cell deaths (Parajuli et al., 2015). This cascade constitutes a key component in the pathophysiology of NE (Deng et al., 2008; Kaur et al., 2013; McRae et al., 1995; Rathnasamy et al., 2011; Sivakumar et al., 2010). In together with our finding, these studies suggest that eotaxin-1 may participate in the evolvement of NE brain injury.

Both circulating and brain tissue levels of eotaxin-1 were found increased following traumatic brain injury in adult mouse model (Shein et al., 2014). In mouse models of adult stroke, eotaxin-1 was elevated following ischemic insult, and appeared to exacerbate acute brain injury and short-term neurological impairment (Lieschke et al., 2019; Roy-O'Reilly et al., 2017). Moreover, in the same model, blockade of eotaxin-1 signaling successfully reversed the aggravation of brain injury by eotaxin-1, by increasing neuronal densities and reducing infarct volumes (Lieschke et al., 2019). These current studies were in line with our finding that eotaxin-1 plays a role in brain injury pathogenesis, and presented eotaxin-1 as an attractive therapeutic target. Conversely, lower serum level of eotaxin-1 was found associated with increased severity

and poorer functional outcomes in human stroke patients, contradicting the findings in animal stroke models (Roy-O'Reilly et al., 2017). In a P9 mouse model of NE, eotaxin-1 demonstrated an increase at the injury site where NPCs migrated towards, and it was further confirmed in vitro that eotaxin-1 promoted NPC proliferation and migration, suggesting a potential neurorestorative effect of eotaxin-1 (Wang et al., 2017). While another in vitro study on human NPCs showed that eotaxin-1 inhibited the proliferation of NPC (Krathwohl & Kaiser, 2004).

PARC, also known as CCL18, is a chemokine expressed by leukocytes, in particular monocytes and dendritic cells (Schutyser et al., 2005), as well as microglia, astrocytes, and neurons in the CNS (Chang et al., 2010). Its production has been shown to be elevated under inflammatory conditions (Schutyser et al., 2005). In addition to attracting T lymphocytes, T-helper-2 (Th2) cells, and B lymphocytes that sustain inflammation (Chang et al., 2010; Schraufstatter et al., 2012), PARC has also been shown to recruit regulatory T cells to suppress neuroinflammatory responses and maintain homeostasis (Chenivesse et al., 2012; González-Maya & González-Barrios, 2021). The regulatory role of PARC in inflammation also lies in inducing anti-inflammatory M2 phenotype of macrophages (Schraufstatter et al., 2012), and inhibiting chemotactic activities of various other chemokines by competitively binding to their receptors (Krohn et al., 2013). Enhanced level of PARC in biofluids has been found in a variety of pathological and inflammatory conditions from cancers to joint, lung, and skin diseases (Schutyser et al., 2005). In CNS diseases, increased plasma level of PARC was associated with more severe inflammatory and neurodegenerative MRI outcomes in 138 multiple sclerosis (MS) adult patients (Ziliotto et al., 2018). Nonetheless, the body fluid levels of PARC were reported to reflect opposite sequels in neonatal brain injuries. In a study that involved 10 pairs of preterm neonates with CP and healthy controls, lower cord blood level of PARC demonstrated significant

association with neonates who developed CP (Kaukola et al., 2004). The lower cord blood level of PARC also robustly predicted intraventricular hemorrhage in 163 preterm neonates (Kallankari et al., 2010). The different expression levels of PARC in adult and neonatal brain injury suggested that the developing and mature brains may have distinct coping mechanisms against injury. The exact role of PARC in the development of brain injury in NE requires further research. In our finding of increased plasma level of PARC in neonates who developed brain injury, it is possible that PARC contributed to the brain injury by inducing chemotaxis, or was upregulated in order to help return to homeostasis.

It is interesting to see that each marker we found enhanced in neonates who developed brain injury seems to have both deleterious and protective effects, considering that neuroinflammatory responses have been acknowledged to have dual roles in brain injury (Ceulemans et al., 2010). Moreover, NE is an active process (Chakkarapani et al., 2021; Davidson et al., 2021), and so is inflammation. Inflammation responders such as immune cells and cytokines are critical for suppressing inflammation initially; however, when the inflammatory conditions remain unresolved, secondary damages and/or persistent cytotoxicity may occur and constantly disrupt the return to homeostasis (Bernis et al., 2022; Fleiss et al., 2021). Hence, the interpretations of the inflammatory biomarker levels should be closely related to the timing of the observations (Ceulemans et al., 2010; Murray, D. M., 2019).

We measure the inflammatory protein levels in plasma on day 2 of life, which is in the middle of the TH treatment. The advantage of this timing is that the biomarker levels we obtained can in a certain degree reflect the impact of TH on them. The biomarkers we found associated with brain injury might be the components that TH mediated inadequately and thus may represent potential therapeutic targets. Alternatively, these elevated inflammatory proteins

could also be part of the protective mechanisms activated early in respond to the brain injury, although their persistent high level may result in unwanted damage. Further research on their evolution of plasma levels over time may help test this hypothesis.

Our study presents certain limitations. One of them is the very limited number of patients with NE treated with TH who did not have brain injury. Further validating research should be conducted before generalizing our results to larger population. Another limitation is the single timing of measurement, whereas serial sampling may better provide a dynamic profile of these markers and enhance the time-dependant predictive power for the brain injury.

2.6 Conclusion

In conclusion, this study provided insight into the link between plasma inflammatory biomarkers and NE brain injury. It revealed a potential association between the early upregulated IL-12p40, SCF, eotaxin-1, PARC, and the eventual brain injury development in neonates with NE who received TH. These inflammatory biomarkers may help assess the risk for brain injury development, and may also represent potential therapeutic targets for adjunctive treatments.

2.7 References

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. *Developmental neuroscience*, *39*(1-4), 59–65. https://doi.org/10.1159/000471508

al Naqeeb, N., Edwards, A. D., Cowan, F. M., & Azzopardi, D. (1999). Assessment of neonatal encephalopathy by amplitude-integrated electroencephalography. Pediatrics, 103(6 Pt 1), 1263–1271. https://doi.org/10.1542/peds.103.6.1263

Al-Macki, N., Miller, S. P., Hall, N., & Shevell, M. (2009). The spectrum of abnormal neurologic outcomes subsequent to term intrapartum asphyxia. *Pediatric neurology*, *41*(6), 399–405. https://doi.org/10.1016/j.pediatrneurol.2009.06.001

Aloisi, F., Penna, G., Cerase, J., Menéndez Iglesias, B., & Adorini, L. (1997). IL-12 production by central nervous system microglia is inhibited by astrocytes. *Journal of immunology (Baltimore, Md. : 1950)*, *159*(4), 1604–1612.

Aslam, S., Strickland, T., & Molloy, E. J. (2019). Neonatal Encephalopathy: Need for Recognition of Multiple Etiologies for Optimal Management. *Frontiers in Pediatrics, 7*.

Azzopardi, D. V., Strohm, B., Edwards, A. D., Dyet, L., Halliday, H. L., Juszczak, E., Kapellou, O., Levene, M., Marlow, N., Porter, E., Thoresen, M., Whitelaw, A., Brocklehurst, P., & TOBY Study Group (2009). Moderate hypothermia to treat perinatal asphyxial encephalopathy. *The New England journal of medicine*, *361*(14), 1349–1358. https://doi.org/10.1056/NEJMoa0900854

Barkovich, A. J., Hajnal, B. L., Vigneron, D., Sola, A., Partridge, J. C., Allen, F., & Ferriero, D. M. (1998). Prediction of neuromotor outcome in perinatal asphyxia: evaluation of MR scoring systems. *AJNR. American journal of neuroradiology*, *19*(1), 143–149.

Bartha, A. I., Foster-Barber, A., Miller, S. P., Vigneron, D. B., Glidden, D. V., Barkovich, A. J., & Ferriero, D. M. (2004). Neonatal Encephalopathy: Association of Cytokines with MR Spectroscopy and Outcome. *Pediatric Research, 56*(6), 960-966. doi:10.1203/01.PDR.0000144819.45689.BB

Baruch, K., Ron-Harel, N., Gal, H., Deczkowska, A., Shifrut, E., Ndifon, W., . . . Schwartz, M. (2013). CNS-specific immunity at the choroid plexus shifts toward destructive Th2 inflammation in brain aging. *Proceedings of the National Academy of Sciences, 110*(6), 2264-2269. doi:10.1073/pnas.1211270110

Bernis, M. E., Schleehuber, Y., Zweyer, M., Maes, E., Felderhoff-Müser, U., Picard, D., & Sabir, H. (2022). Temporal Characterization of Microglia-Associated Pro- and Anti-Inflammatory Genes in a Neonatal Inflammation-Sensitized Hypoxic-Ischemic Brain Injury Model. *Oxidative Medicine and Cellular Longevity, 2022*, 2479626. doi:10.1155/2022/2479626

Bona, E., Andersson, A.-L., Blomgren, K., Gilland, E., Puka-Sundvall, M., Gustafson, K., & Hagberg, H. (1999). Chemokine and Inflammatory Cell Response to Hypoxia-Ischemia in Immature Rats. *Pediatric Research, 45*(4), 500-509. doi:10.1203/00006450-199904010-00008

Boudes, E., Tan, X., Saint-Martin, C., Shevell, M., & Wintermark, P. (2015). MRI obtained during versus after hypothermia in asphyxiated newborns. *Archives of Disease in*

Childhood - Fetal and Neonatal Edition, 100(3), F238. doi:10.1136/archdischild-2014-306550

Bryce, J., Boschi-Pinto, C., Shibuya, K., Black, R. E., & WHO Child Health Epidemiology Reference Group (2005). WHO estimates of the causes of death in children. *Lancet (London, England)*, *365*(9465), 1147–1152. https://doi.org/10.1016/S0140-6736(05)71877-8

Ceulemans, A. G., Zgavc, T., Kooijman, R., Hachimi-Idrissi, S., Sarre, S., & Michotte, Y. (2010). The dual role of the neuroinflammatory response after ischemic stroke: modulatory effects of hypothermia. *Journal of neuroinflammation*, *7*, 74. https://doi.org/10.1186/1742-2094-7-74

Chakkarapani, A. A., Aly, H., Benders, M., Cotten, C. M., El-Dib, M., Gressens, P., . . . Robertson, N. J. (2021). Therapies for neonatal encephalopathy: Targeting the latent, secondary and tertiary phases of evolving brain injury. *Seminars in Fetal and Neonatal Medicine, 26*(5), 101256. doi:https://doi.org/10.1016/j.siny.2021.101256

Chalak L. F. (2016). Inflammatory Biomarkers of Birth Asphyxia. Clinics in perinatology, 43(3), 501–510. https://doi.org/10.1016/j.clp.2016.04.008

Chalak, L. F., Sánchez, 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. *The Journal of pediatrics*, *164*(3), 468–74.e1. https://doi.org/10.1016/j.jpeds.2013.10.067

Chang, C. Y., Lee, Y. H., Leu, S. J., Wang, C. Y., Wei, C. P., Hung, K. S., Pai, M. H., Tsai, M. D., & Wu, C. H. (2010). CC-chemokine ligand 18/pulmonary activation-regulated chemokine expression in the CNS with special reference to traumatic brain injuries and

neoplastic disorders. Neuroscience, 165(4), 1233–1243. https://doi.org/10.1016/j.neuroscience.2009.11.050

Chenivesse, C., Chang, Y., Azzaoui, I., Ait Yahia, S., Morales, O., Plé, C., . . . Tsicopoulos, A. (2012). Pulmonary CCL18 Recruits Human Regulatory T Cells. *The Journal of Immunology, 189*(1), 128. doi:10.4049/jimmunol.1003616

Cheong, J. L. Y., Coleman, L., Hunt, R. W., Lee, K. J., Doyle, L. W., Inder, T. E., . . . Infant Cooling Evaluation Collaboration, f. t. (2012). Prognostic Utility of Magnetic Resonance Imaging in Neonatal Hypoxic-Ischemic Encephalopathy: Substudy of a Randomized Trial. *Archives of Pediatrics & Adolescent Medicine, 166*(7), 634-640. doi:10.1001/archpediatrics.2012.284

Cooper, A. M., & Khader, S. A. (2007). IL-12p40: an inherently agonistic cytokine. *Trends in Immunology, 28*(1), 33-38. doi:https://doi.org/10.1016/j.it.2006.11.002

Davidson, J. O., Gonzalez, F., Gressens, P., & Gunn, A. J. (2021). Update on mechanisms of the pathophysiology of neonatal encephalopathy. *Seminars in Fetal and Neonatal Medicine, 26*(5), 101267. doi:https://doi.org/10.1016/j.siny.2021.101267

Deng, Y., Lu, J., Sivakumar, V., Ling, E. A., & Kaur, C. (2008). Amoeboid microglia in the periventricular white matter induce oligodendrocyte damage through expression of proinflammatory cytokines via MAP kinase signaling pathway in hypoxic neonatal rats. *Brain pathology (Zurich, Switzerland)*, *18*(3), 387–400. https://doi.org/10.1111/j.1750-3639.2008.00138.x

Doycheva, D., Shih, G., Chen, H., Applegate, R., Zhang, J. H., & Tang, J. (2013). Granulocyte-colony stimulating factor in combination with stem cell factor confers greater neuroprotection after hypoxic-ischemic brain damage in the neonatal rats than a solitary

treatment. *Translational stroke research*, *4*(2), 171–178. https://doi.org/10.1007/s12975-012-0225-2

Dumbuya, J. S., Chen, L., Wu, J.-Y., & Wang, B. (2021). The role of G-CSF neuroprotective effects in neonatal hypoxic-ischemic encephalopathy (HIE): current status. *Journal of Neuroinflammation, 18*(1), 55. doi:10.1186/s12974-021-02084-4

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

Fleiss, B., Van Steenwinckel, J., Bokobza, C., K Shearer, I., Ross-Munro, E., & Gressens, P. (2021). Microglia-Mediated Neurodegeneration in Perinatal Brain Injuries. *Biomolecules*, *11*(1), 99. https://doi.org/10.3390/biom11010099

Gately, M. K., Carvajal, D. M., Connaughton, S. E., Gillessen, S., Warrier, R. R., Kolinsky, K. D., . . . Presky, D. H. (1996). Interleukin-12 Antagonist Activity of Mouse Interleukin-12 p40 Homodimer in Vitro and in Vivo. *Annals of the New York Academy of Sciences, 795*(1), 1-12. doi:https://doi.org/10.1111/j.1749-6632.1996.tb52650.x

Gee, K., Guzzo, C., Che Mat, N. F., Ma, W., & Kumar, A. (2009). The IL-12 family of cytokines in infection, inflammation and autoimmune disorders. *Inflammation & allergy drug targets*, *8*(1), 40–52. https://doi.org/10.2174/187152809787582507

Gillessen, S., Carvajal, D., Ling, P., Podlaski, F. J., Stremlo, D. L., Familletti, P. C., . . . Gately, M. K. (1995). Mouse interleukin-12 (IL-12) p40 homodimer: a potent IL-12 antagonist. *European Journal of Immunology, 25*(1), 200-206. doi:https://doi.org/10.1002/eji.1830250133

Gluckman, P. D., Wyatt, J. S., Azzopardi, D., Ballard, R., Edwards, A. D., Ferriero, D. M., Polin, R. A., Robertson, C. M., Thoresen, M., Whitelaw, A., & Gunn, A. J. (2005). Selective head cooling with mild systemic hypothermia after neonatal encephalopathy: multicentre randomised trial. *Lancet (London, England)*, *365*(9460), 663–670. https://doi.org/10.1016/S0140-6736(05)17946-X

Go, H., Saito, Y., Maeda, H., Maeda, R., Yaginuma, K., Ogasawara, K., Kashiwabara, N., Kawasaki, Y., & Hosoya, M. (2021). Serum cytokine profiling in neonates with hypoxic ischemic encephalopathy. *Journal of neonatal-perinatal medicine*, *14*(2), 177–182. https://doi.org/10.3233/NPM-200431

González-Maya, M., & González-Barrios, J. A. (2021). Chapter 4 - Single-nucleotide polymorphisms and cerebral palsy. In C. R. Martin, V. R. Preedy, & R. Rajendram (Eds.), *Factors Affecting Neurodevelopment* (pp. 39-47): Academic Press.

Ha, S. J., Lee, C. H., Lee, S. B., Kim, C. M., Jang, K. L., Shin, H. S., & Sung, Y. C. (1999). A Novel Function of IL-12p40 as a Chemotactic Molecule for Macrophages. *The Journal of Immunology, 163*(5), 2902.

Hagberg, H., Gilland, E., Bona, E., Hanson, L. A., Hahin-Zoric, M., Blennow, M., Holst, M., McRae, A., & Söder, O. (1996). Enhanced expression of interleukin (IL)-1 and IL-6 messenger RNA and bioactive protein after hypoxia-ischemia in neonatal rats. *Pediatric research*, *40*(4), 603–609. https://doi.org/10.1203/00006450-199610000-00015

Hassell, K. J., Ezzati, M., Alonso-Alconada, D., Hausenloy, D. J., & Robertson, N. J. (2015). New horizons for newborn brain protection: enhancing endogenous neuroprotection. *Archives of disease in childhood. Fetal and neonatal edition*, *100*(6), F541–F552. https://doi.org/10.1136/archdischild-2014-306284

He, M. L., Lv, Z. Y., Shi, X., Yang, T., Zhang, Y., Li, T. Y., & Chen, J. (2017). Interleukin-10 release from astrocytes suppresses neuronal apoptosis via the TLR2/NFκB pathway in a neonatal rat model of hypoxic-ischemic brain damage. *Journal of neurochemistry*, *142*(6), 920–933. https://doi.org/10.1111/jnc.14126

Hedtjärn, M., Leverin, A. L., Eriksson, K., Blomgren, K., Mallard, C., & Hagberg, H. (2002). Interleukin-18 involvement in hypoxic-ischemic brain injury. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *22*(14), 5910–5919. https://doi.org/10.1523/JNEUROSCI.22-14-05910.2002

Jiao, M., Li, X., Chen, L., Wang, X., Yuan, B., Liu, T., Dong, Q., Mei, H., & Yin, H. (2020). Neuroprotective effect of astrocyte-derived IL-33 in neonatal hypoxic-ischemic brain injury. *Journal of neuroinflammation*, *17*(1), 251. https://doi.org/10.1186/s12974-020-01932-z

Jin, Y., Silverman, A. J., & Vannucci, S. J. (2009). Mast cells are early responders after hypoxia-ischemia in immature rat brain. *Stroke*, *40*(9), 3107–3112. https://doi.org/10.1161/STROKEAHA.109.549691

Kallankari, H., Kaukola, T., Ojaniemi, M., Herva, R., Perhomaa, M., Vuolteenaho, R., Kingsmore, S. F., & Hallman, M. (2010). Chemokine CCL18 predicts intraventricular hemorrhage in very preterm infants. *Annals of medicine*, *42*(6), 416–425. https://doi.org/10.3109/07853890.2010.481085

Kaukola, T., Satyaraj, E., Patel, D. D., Tchernev, V. T., Grimwade, B. G., Kingsmore, S. F., Koskela, P., Tammela, O., Vainionpää, L., Pihko, H., Äärimaa, T., & Hallman, M. (2004). Cerebral palsy is characterized by protein mediators in cord serum. *Annals of neurology*, *55*(2), 186–194. https://doi.org/10.1002/ana.10809

Keller, M., Simbruner, G., Górna, A., Urbanek, M., Tinhofer, I., Griesmaier, E., . . . Gressens, P. (2006). Systemic Application of Granulocyte-Colony Stimulating Factor and Stem Cell Factor Exacerbates Excitotoxic Brain Injury in Newborn Mice. *Pediatric Research, 59*(4), 549-553. doi:10.1203/01.pdr.0000205152.38692.81

Krathwohl, M. D., & Kaiser, J. L. (2004). Chemokines Promote Quiescence and Survival of Human Neural Progenitor Cells. *Stem Cells, 22*(1), 109-118. doi:10.1634/stemcells.22-1-109

Krohn, S. C., Bonvin, P., & Proudfoot, A. E. (2013). CCL18 exhibits a regulatory role through inhibition of receptor and glycosaminoglycan binding. *PloS one*, *8*(8), e72321. https://doi.org/10.1371/journal.pone.0072321

Lau, L. T., & Yu, A. C. (2001). Astrocytes produce and release interleukin-1, interleukin-6, tumor necrosis factor alpha and interferon-gamma following traumatic and metabolic injury. *Journal of neurotrauma*, *18*(3), 351–359. https://doi.org/10.1089/08977150151071035

Li, S. J., Liu, W., Wang, J. L., Zhang, Y., Zhao, D. J., Wang, T. J., & Li, Y. Y. (2014). The role of TNF-α, IL-6, IL-10, and GDNF in neuronal apoptosis in neonatal rat with hypoxic-ischemic encephalopathy. *European review for medical and pharmacological sciences*, *18*(6), 905–909.

Lieschke, S., Zechmeister, B., Haupt, M., Zheng, X., Jin, F., Hein, K., Weber, M. S., Hermann, D. M., Bähr, M., Kilic, E., & Doeppner, T. R. (2019). CCL11 Differentially Affects Post-Stroke Brain Injury and Neuroregeneration in Mice Depending on Age. *Cells*, *9*(1), 66. https://doi.org/10.3390/cells9010066

Liu, F., & McCullough, L. D. (2013). Inflammatory responses in hypoxic ischemic encephalopathy. *Acta Pharmacologica Sinica, 34*(9), 1121-1130. doi:10.1038/aps.2013.89

Mattner, F., Fischer, S., Guckes, S., Jin, S., Kaulen, H., Schmitt, E., Rüde, E., & Germann, T. (1993). The interleukin-12 subunit p40 specifically inhibits effects of the interleukin-12 heterodimer. *European journal of immunology*, *23*(9), 2202–2208. https://doi.org/10.1002/eji.1830230923

McGowan, M. M., O'Kane, A. C., Vezina, G., Chang, T., Bendush, N., Glass, P., Gai, J., Bost, J., Everett, A. D., & Massaro, A. N. (2021). Serial plasma biomarkers of brain injury in infants with neonatal encephalopathy treated with therapeutic hypothermia. *Pediatric research*, *90*(6), 1228–1234. https://doi.org/10.1038/s41390-021-01405-w

Montaldo, P., Kaforou, M., Pollara, G., Hervás-Marín, D., Calabria, I., Panadero, J., Pedrola, L., Lally, P. J., Oliveira, V., Kage, A., Atreja, G., Mendoza, J., Soe, A., Pattnayak, S., Shankaran, S., Vento, M., Herberg, J., & Thayyil, S. (2019). Whole Blood Gene Expression Reveals Specific Transcriptome Changes in Neonatal Encephalopathy. *Neonatology*, *115*(1), 68–76. https://doi.org/10.1159/000492420

Murray, D. M. (2019). Chapter 13 - Biomarkers in neonatal hypoxic–ischemic encephalopathy—Review of the literature to date and future directions for research. In L. S. de Vries & H. C. Glass (Eds.), *Handbook of Clinical Neurology* (Vol. 162, pp. 281-293): Elsevier.

Nair, J., & Kumar, V. (2018). Current and Emerging Therapies in the Management of Hypoxic Ischemic Encephalopathy in Neonates. *Children (Basel, Switzerland)*, *5*(7), 99. https://doi.org/10.3390/children5070099

Neubauer, V., Wegleiter, K., Posod, A., Urbanek, M., Wechselberger, K., Kiechl-Kohlendorfer, U., Keller, M., & Griesmaier, E. (2016). Delayed application of the haematopoietic growth factors G-CSF/SCF and FL reduces neonatal excitotoxic brain injury. *Brain research*, *1634*, 94–103. https://doi.org/10.1016/j.brainres.2015.12.058

Nilsson, G., Butterfield, J. H., Nilsson, K., & Siegbahn, A. (1994). Stem cell factor is a chemotactic factor for human mast cells. *The Journal of Immunology, 153*(8), 3717.

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

Parajuli, B., Horiuchi, H., Mizuno, T., Takeuchi, H., & Suzumura, A. (2015). CCL11 enhances excitotoxic neuronal death by producing reactive oxygen species in microglia. *Glia*, *63*(12), 2274–2284. https://doi.org/10.1002/glia.22892

Posod, A., Wegleiter, K., Neubauer, V., Urbanek, M., Huber, E., Kiechl-Kohlendorfer, U., Keller, M., & Griesmaier, E. (2019). Short-, Mid-, and Long-Term Effect of Granulocyte Colony-Stimulating Factor/Stem Cell Factor and Fms-Related Tyrosine Kinase 3 Ligand Evaluated in an In Vivo Model of Hypoxic-Hyperoxic Ischemic Neonatal Brain Injury. *BioMed research international*, *2019*, 5935279. https://doi.org/10.1155/2019/5935279

Reber, L., Da Silva, C. A., & Frossard, N. (2006). Stem cell factor and its receptor c-Kit as targets for inflammatory diseases. *European Journal of Pharmacology, 533*(1), 327-340. doi:https://doi.org/10.1016/j.ejphar.2005.12.067

Roy-O'Reilly, M., Ritzel, R. M., Conway, S. E., Staff, I., Fortunato, G., & McCullough, L. D. (2017). CCL11 (Eotaxin-1) Levels Predict Long-Term Functional Outcomes in Patients Following Ischemic Stroke. *Translational stroke research*, *8*(6), 578–584. https://doi.org/10.1007/s12975-017-0545-3

Russell, T. D., Yan, Q., Fan, G., Khalifah, A. P., Bishop, D. K., Brody, S. L., & Walter, M. J. (2003). IL-12 p40 Homodimer-Dependent Macrophage Chemotaxis and Respiratory Viral Inflammation Are Mediated through IL-12 Receptor β1. *The Journal of Immunology, 171*(12), 6866. doi:10.4049/jimmunol.171.12.6866

Sävman, K., Blennow, M., Gustafson, K., Tarkowski, E., & Hagberg, H. (1998). Cytokine response in cerebrospinal fluid after birth asphyxia. *Pediatric research*, *43*(6), 746–751. https://doi.org/10.1203/00006450-199806000-00006

Schraufstatter, I. U., Zhao, M., Khaldoyanidi, S. K., & Discipio, R. G. (2012). The chemokine CCL18 causes maturation of cultured monocytes to macrophages in the M2 spectrum. Immunology, 135(4), 287–298. https://doi.org/10.1111/j.1365-2567.2011.03541.x

Schutyser, E., Richmond, A., & Van Damme, J. (2005). Involvement of CC chemokine ligand 18 (CCL18) in normal and pathological processes. Journal of leukocyte biology, 78(1), 14–26. https://doi.org/10.1189/jlb.1204712

Shalak, L., & Perlman, J. M. (2004). Hypoxic–ischemic brain injury in the term infant-current concepts. *Early Human Development, 80*(2), 125-141. doi:https://doi.org/10.1016/j.earlhumdev.2004.06.003

Shankaran S. (2012). Therapeutic hypothermia for neonatal encephalopathy. *Current treatment options in neurology*, *14*(6), 608–619. https://doi.org/10.1007/s11940-012-0200-y

Shankaran, S., Laptook, A. R., Ehrenkranz, R. A., Tyson, J. E., McDonald, S. A., Donovan, E. F., Fanaroff, A. A., Poole, W. K., Wright, L. L., Higgins, R. D., Finer, N. N., Carlo, W. A., Duara, S., Oh, W., Cotten, C. M., Stevenson, D. K., Stoll, B. J., Lemons, J. A., Guillet, R., Jobe, A. H., … National Institute of Child Health and Human Development Neonatal Research Network (2005). Whole-body hypothermia for neonates with hypoxic-ischemic encephalopathy. *The New England journal of medicine*, *353*(15), 1574–1584. https://doi.org/10.1056/NEJMcps050929

Shein, S. L., Shellington, D. K., Exo, J. L., Jackson, T. C., Wisniewski, S. R., Jackson, E. K., Vagni, V. A., Bayır, H., Clark, R. S., Dixon, C. E., Janesko-Feldman, K. L., & Kochanek, P. M. (2014). Hemorrhagic shock shifts the serum cytokine profile from pro- to anti-inflammatory after experimental traumatic brain injury in mice. *Journal of neurotrauma*, *31*(16), 1386–1395. https://doi.org/10.1089/neu.2013.2985

Sivakumar, V., Ling, E. A., Lu, J., & Kaur, C. (2010). Role of glutamate and its receptors and insulin-like growth factors in hypoxia induced periventricular white matter injury. *Glia*, *58*(5), 507–523. https://doi.org/10.1002/glia.20940

Szaflarski, J., Burtrum, D., & Silverstein, F. S. (1995). Cerebral hypoxia-ischemia stimulates cytokine gene expression in perinatal rats. *Stroke*, *26*(6), 1093–1100. https://doi.org/10.1161/01.str.26.6.1093

Teixeira, A. L., Gama, C. S., Rocha, N. P., & Teixeira, M. M. (2018). Revisiting the Role of Eotaxin-1/CCL11 in Psychiatric Disorders. *Frontiers in Psychiatry, 9*.

Trinchieri, G. (2003). Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nature Reviews Immunology, 3*(2), 133-146. doi:10.1038/nri1001

Vasiljevic, B., Maglajlic-Djukic, S., Gojnic, M., Stankovic, S., Ignjatovic, S., & Lutovac, D. (2011). New insights into the pathogenesis of perinatal hypoxic-ischemic brain injury. *Pediatrics international : official journal of the Japan Pediatric Society*, *53*(4), 454–462. https://doi.org/10.1111/j.1442-200X.2010.03290.x

Vignali, D. A. A., & Kuchroo, V. K. (2012). IL-12 family cytokines: immunological playmakers. *Nature Immunology, 13*(8), 722-728. doi:10.1038/ni.2366

Volpe J. J. (2012). Neonatal encephalopathy: an inadequate term for hypoxic-ischemic encephalopathy. *Annals of neurology*, *72*(2), 156–166. https://doi.org/10.1002/ana.23647

Wakabayashi, K., Isozaki, T., Tsubokura, Y., Fukuse, S., & Kasama, T. (2021). Eotaxin-1/CCL11 is involved in cell migration in rheumatoid arthritis. *Scientific Reports, 11*(1), 7937. doi:10.1038/s41598-021-87199-7

Walsh, B. H., Boylan, G. B., Livingstone, V., Kenny, L. C., Dempsey, E. M., & Murray, D. M. (2013). Cord Blood Proteins and Multichannel-Electroencephalography in Hypoxic-Ischemic Encephalopathy*. *Pediatric Critical Care Medicine, 14*(6).

Walter, M. J., Kajiwara, N., Karanja, P., Castro, M., & Holtzman, M. J. (2001). Interleukin 12 P40 Production by Barrier Epithelial Cells during Airway Inflammation. *Journal of Experimental Medicine, 193*(3), 339-352. doi:10.1084/jem.193.3.339

Wang, F., Baba, N., Shen, Y., Yamashita, T., Tsuru, E., Tsuda, M., . . . Sagara, Y. (2017). CCL11 promotes migration and proliferation of mouse neural progenitor cells. *Stem Cell Research & Therapy, 8*(1), 26. doi:10.1186/s13287-017-0474-9

Wintermark, P. (2011). Current Controversies in Newer Therapies to Treat Birth Asphyxia. *International Journal of Pediatrics, 2011*, 848413. doi:10.1155/2011/848413

Wintermark, P., Hansen, A., Soul, J., Labrecque, M., Robertson, R. L., & Warfield, S. K. (2011). Early versus late MRI in asphyxiated newborns treated with hypothermia. *Archives of disease in childhood. Fetal and neonatal edition*, *96*(1), F36–F44. https://doi.org/10.1136/adc.2010.184291

Wisnowski, J. L., Wintermark, P., Bonifacio, S. L., Smyser, C. D., Barkovich, A. J., Edwards, A. D., de Vries, L. S., Inder, T. E., Chau, V., & Newborn Brain Society Guidelines and Publications Committee (2021). Neuroimaging in the term newborn with neonatal encephalopathy. Seminars in fetal & neonatal medicine, 26(5), 101304. https://doi.org/10.1016/j.siny.2021.101304

Wood, T. R., Vu, P. T., Comstock, B. A., Law, J. B., Mayock, D. E., Heagerty, P. J., Burbacher, T., Bammler, T. K., & Juul, S. E. (2021). Cytokine and chemokine responses to injury and treatment in a nonhuman primate model of hypoxic-ischemic encephalopathy treated with hypothermia and erythropoietin. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*, *41*(8), 2054–2066. https://doi.org/10.1177/0271678X21991439

Zhao, L. R., Piao, C. S., Murikinati, S. R., & Gonzalez-Toledo, M. E. (2013). The role of stem cell factor and granulocyte-colony stimulating factor in treatment of stroke. *Recent patents on CNS drug discovery*, *8*(1), 2–12. https://doi.org/10.2174/1574889811308010002

Ziliotto, N., Bernardi, F., Jakimovski, D., Baroni, M., Bergsland, N., Ramasamy, D. P., . . . Ramanathan, M. (2018). Increased CCL18 plasma levels are associated with neurodegenerative MRI outcomes in multiple sclerosis patients. *Multiple Sclerosis and Related Disorders, 25*, 37-42. doi:https://doi.org/10.1016/j.msard.2018.07.009

2.8 Figures

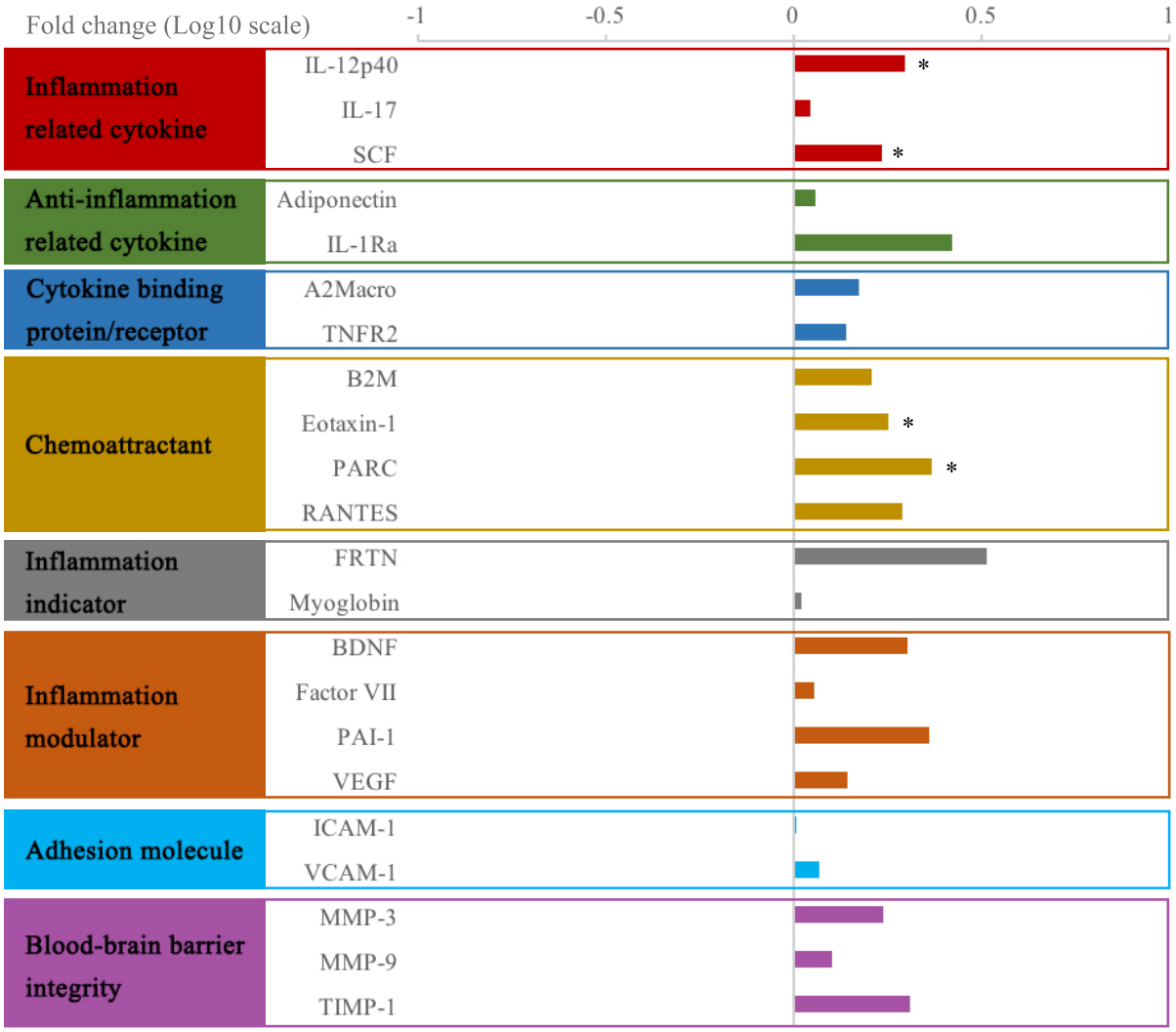

Figure 1. Expression of inflammation-related protein markers on day 2 of life. Mean protein concentrations were compared between near-term and term neonates with NE treated with TH (Group 1) without brain injury and (Group 2) with brain injury. *p* values were calculated to highlight significant protein expression differences between the groups (* $p < 0.05$). For graphical representation, ratios of the Group 2 (with brain injury) mean concentration over the Group 1 (without brain injury) mean concentration were calculated and log transformed to obtain fold change data for each marker.

2.9 Tables

Table 1. Inflammation-related protein markers analyzed

Inflammation related cytokine	**Chemoattractant**	**Adhesion molecule**
Interleukin-1 alpha (IL-1 alpha)	Beta-2-Microglobulin (B2M)	Intercellular Adhesion Molecule 1 (ICAM-1)
Interleukin-1 beta (IL-1 beta)	Eotaxin-1	Vascular Cell Adhesion Molecule-1 (VCAM-1)
Interleukin-12 Subunit p40 (IL-12p40)	Pulmonary and Activation-Regulated Chemokine (PARC)	
Interleukin-12 Subunit p70 (IL-12p70)	T-Cell-Specific Protein RANTES (RANTES)	**Blood-brain barrier integrity**
Interleukin-17 (IL-17)		Matrix Metalloproteinase-3 (MMP-3)
Stem Cell Factor (SCF)	**Inflammation indicator**	Matrix Metalloproteinase-9 (MMP-9)
	Ferritin (FRTN)	Tissue Inhibitor of Metalloproteinases 1 (TIMP-1)
Anti-inflammation related cytokine	Myoglobin	
Adiponectin		
Interleukin-1 receptor antagonist (IL-1ra)	**Inflammation modulator**	
	Brain-Derived Neurotrophic Factor (BDNF)	
	Factor VII	
Cytokine binding protein/receptor	Plasminogen Activator Inhibitor 1 (PAI-1)	
Alpha-2-Macroglobulin (A2Macro)	Vascular Endothelial Growth Factor (VEGF)	
Tumor necrosis factor receptor 2 (TNFR2)		

The 25 proteins analyzed are categorized based on their predominant role in inflammation. Many of these proteins may be multifunctional and may fit into more than one category.

Table 2. Clinical characteristics of study patients

Genearal characteristic	All neonates with NE treated with TH (n = 35)	Neonates with NE treated with TH without MRI evidence of brain injury (n = 5)	Neonates with NE treated with TH with MRI evidence of brain injury (n = 30)	*P* value
Gestational age (w), mean ± SD	39.49 ± 1.53	38.74 ± 2.02	39.61 ± 1.44	*0.34*
Birth weight (g), mean ± SD	3181 ± 820.9	3263 ± 277.3	3167 ± 882.1	*0.83*
Gender, n (%)				*0.59*
Male	26 (74)	3 (60)	23 (77)	
Female	9 (26)	2 (40)	7 (23)	
Apgar score ≤ 5 at 10 minutes, n (%)	24 (73)	3 (60)	21 (75)	*0.60*
Arterial cord pH, mean ± SD	7.01 ± 0.17	7.08 ± 0.13	6.99 ± 0.18	*0.49*
Initial postnatal blood gas pH, mean ± SD	7.04 ± 0.23	7.08 ± 0.12	7.03 ± 0.24	*0.30*
Initial modified Sarnat score on admission, n (%)				*0.30*
1	2 (6)	1 (20)	1 (3)	
2	21 (60)	3 (60)	18 (60)	
3	12 (34)	1 (20)	11 (37)	
Initial aEEG background, n (%)				*0.13*
Normal	0 (0)	0 (0)	0 (0)	
Moderately abnormal	14 (40)	4 (80)	10 (33)	
Severely abnormal	21 (60)	1 (20)	20 (67)	
Death, n (%)	7 (20)	0 (0)	7 (23)	*0.56*
BG/W score, n (%)				*< 0.0001*
0	6 (17)	5 (100)	1 (3)	
1	3 (9)	0 (0)	3 (10)	
2	7 (20)	0 (0)	7 (23)	
3	9 (26)	0 (0)	9 (30)	
4	10 (29)	0 (0)	10 (33)	
WBC on DOL2 (10^9/L), mean ± SD	13.37 ± 6.203	9.585 ± 2.120	13.91 ± 6.423	*0.12*
Placental pathology, n (%)				*0.86*
Vilitis of unknown etiology	4 (13)	0 (0)	4 (14)	
Chorioamnionitis	10 (32)	1 (33)	9 (32)	
Chorioamnionitis with fetal vasculitis	7 (23)	1 (33)	6 (21)	
Chorioamnionitis without fetal vasculitis	1 (3)	0 (0)	1 (4)	

aEEG, amplitude-integrated electroencephalogram; *BG/W*, Barkovich MRI basal ganglia/watershed score; *DOL*, day of life; *MRI*, magnetic resonance imaging; *NE*, neonatal encephalopathy; *TH*, therapeutic hypothermia; *WBC*, white blood cell count.

aEEG background activity was defined using previously described classifications. (al Naqeeb et al., 1999)

Chapter 3

Impact of sildenafil on inflammation in neonates with neonatal encephalopathy treated with hypothermia

Ruofan Song, Emmanouil Rampakakis, Pia Wintermark

Status of the Article: In preparation for submission to a journal

Foreword: Blood inflammatory biomarkers have been evidenced to be associated with the severity and/or outcome in NE, and have been utilized to assess the efficacy of therapeutic interventions in NE. In the previous Chapter 2, we identified potential inflammatory biomarkers that may help distinguish the neonates with NE who do not respond adequately to TH and develop brain injury. Those inflammation-related proteins might be involved in the brain injury development process, and thus represent potential therapeutic targets. Thereupon, we were interested to investigate whether sildenafil, a promising adjuvant treatment that has demonstrated inflammation-modulating effects in rat models of NE, has an impact on the inflammatory biomarkers in neonates with NE treated with TH. In this Chapter, we measured the plasma concentrations of a same panel of inflammation-related proteins as in Chapter 2. Brain injury and/or sildenafil appeared to upregulate interleukin (IL)-12p40 and interleukin-1 receptor antagonist (IL-1Ra), and sildenafil appeared to upregulate eotaxin-1 and downregulate alpha-2-macroglobulin (A2Macro). Our findings suggest that sildenafil in addition to TH may modulate inflammation and potentially promote neurorestoration, and may thus represent a promising adjunctive treatment to TH in NE.

3.1 Abstract

Background: To date, neonatal encephalopathy (NE) has only one proven treatment, therapeutic hypothermia (TH). However, many treated neonates still develop brain injury. Neuroinflammation is a key component in the pathophysiology of NE. Mitigating inflammation appears an interesting venue for new adjunctive treatments, and sildenafil has shown inflammation-modulating effects in the rat models of NE.

Objective: This study aimed to investigate the impact of sildenafil on the expression over the first days of life of blood inflammatory biomarkers in neonates with NE treated with TH.

Methods: Plasma samples were collected at different time-points during the first days of life (DOL 2, 3, and 4) in term and near-term neonates with NE treated with TH +/- sildenafil. Presence of brain injury for each neonate were defined by magnetic resonance imaging (MRI). Twenty-five inflammatory markers in the plasma samples were quantified by multi-analyte profiling antigen analysis.

Results: Twenty-nine neonates with NE treated with TH were included in this study, including 5 without brain injury, 11 with brain injury and only treated with TH, and 13 with brain injury and treated with TH and sildenafil. On DOL 3, a lower level of alpha-2-macroglobulin (A2Macro) comparing to the other two groups, and a higher level of interleukin (IL)-12p40 comparing to the no brain injury group were observed in the sildenafil-treated neonates. On DOL 4, a lower level of A2Macro and a higher level of interleukin-1 receptor antagonist (IL-1Ra) comparing to the no brain injury group, and an upregulation of the eotaxin-1 comparing to the other two groups were demonstrated in the sildenafil-treated group.

Conclusion: Sildenafil in addition to TH appears to modulate inflammation, and potentially promotes neurorestoration, and may thus represent a promising adjunctive treatment to TH in NE.

3.2 Introduction

Neonatal encephalopathy (NE) caused by birth asphyxia (Aslam et al., 2019; Hassell et al., 2015; Shalak & Perlman, 2004; Volpe J. J., 2012) has an incidence of 2 to 8 per 1000 live births in high-income countries (Lee et al., 2013) and is responsible for approximately 23% of neonatal deaths globally (Bryce et al., 2005). It leaves surviving children with long-term morbidities, such as cerebral palsy, motor impairments, intellectual disabilities, and epilepsy (Al-Macki et al., 2009; Wintermark, 2011).

Therapeutic hypothermia (TH) is presently the only proven neuroprotective treatment for moderate to severe NE (Shankaran, 2012; Wintermark, 2011; Yazdani et al., 2016), however its efficacy can be limited by its initiation timing and concomitant existence of inflammation (Davidson et al., 2018; Gunn, A. J., & Gunn, T. R., 1998; Nair & Kumar, 2018; Osredkar et al., 2014; Pauliah et al., 2013; Wintermark et al., 2010). In light of that many treated neonates still incur brain injury (Azzopardi et al., 2009; Cheong et al., 2012; Gluckman et al., 2005; Wintermark, 2011), adjuvant therapy to supplement TH is urgently needed to improve the outcomes of neonates with NE. Modulating inflammation appears an interesting venue, given that neuroinflammation has long been acknowledged as a crucial part of NE pathophysiology (Ahearne et al., 2017; Chakkarapani et al., 2021; Fleiss & Gressens, 2012; Nair & Kumar, 2018), and mitigating inflammation appears to contribute to better neurological outcome in the context of NE (Jenkins et al., 2012).

Sildenafil is a promising adjunctive treatment. It is a potent phosphodiesterase type 5 (PDE5) inhibitor that increases the intracellular level of cyclic guanosine monophosphate (cGMP), which may inhibit neuroinflammation (Chakkarapani et al., 2021; Ghofrani et al., 2006; Peixoto et al., 2015). Sildenafil has been reported to have inflammation-modulating effects by

reducing astrocyte and microglia activation, and by modulating inflammatory cytokines in animal models of adult neurological diseases and in rat models of NE (Araújo et al., 2020; Charriaut-Marlangue et al., 2014; Nunes et al., 2015; Yazdani et al., 2021; Zhang et al., 2013). It has been tested in adult patients with stroke (Silver et al., 2009), and is already used safely in neonates with persistent pulmonary hypertension (Simonca & Tulloh, 2017). The impact of sildenafil on inflammation in human neonates with NE is yet to be determined.

Blood inflammatory biomarkers have been actively investigated in clinical settings for their association with severity and/or outcome in neonates with NE treated with TH (Ahearne et al., 2017; Chalak et al., 2014; McGowan et al., 2021; Walsh et al., 2013). Inflammatory markers in biofluids have also been utilized to test the efficacy of new therapeutic approaches (Massaro et al., 2018; Orrock et al., 2016). In this study, we aimed to investigate the impact of sildenafil on inflammation in early days of NE. We hypothesize that sildenafil may keep its inflammation-modulating effects in human neonates with NE treated with TH.

3.3 Method

3.3.1 Patients

Male and female neonates with moderate and severe NE and treated with hypothermia were included in this prospective cohort study. Criteria for TH were those previously described (Azzopardi et al., 2009; Gluckman et al., 2005; Shankaran et al., 2005): (1) gestational age ≥ 36 weeks and birth weight ≥ 1800 grams; (2) evidence of fetal distress, i.e., history of an acute perinatal event, any occurrence of cord pH ≤ 7.0 or base deficit ≤ -16mEq/L; (3) evidence of neonatal distress, i.e., an Apgar score ≤ 5 at 10 minutes after birth, postnatal blood gas pH obtained within the first hour of life ≤ 7.0 or base deficit ≤ -16mEq/L, or a need for ventilation

initiated at birth and continued for at least 10 minutes; and (4) moderate to severe neonatal encephalopathy evidenced by an abnormal neurological exam and/or an aEEG. Eligible neonates were subjected to whole-body cooling to an esophageal temperature of 33.5°C, which began within the first 6 hours of life and continued for 72 hours, and were then slowly rewarmed.

Brain magnetic resonance imaging (MRI) was carried out on day of life 2 (DOL 2) and defined brain injury in a subset of these neonates (Boudes et al., 2015; Wintermark et al., 2011; Wisnowski et al., 2021). Neuroradiologists blinded to the neonates' clinical conditions assessed and scored the MRI results using a previously described MRI scoring system (Barkovich et al., 1998). Neonates who developed brain injury were either treated only with TH or with TH and sildenafil.

Clinical characteristics for each neonate were collected, including gestational age, birth weight, gender, Apgar score at 10 minutes, arterial cord pH, initial postnatal blood gas pH, initial modified Sarnat score on admission, initial aEEG background activity, outcome (i.e., alive or dead), white blood cell (WBC) counts on day 2 of life, and placental pathology results.

The research protocol was approved by the research ethics board from the Montreal Children's Hospital, McGill University Health Centre, and informed written consent was obtained from the parents on behalf of their neonate before any study-specific procedures.

3.3.2 Sildenafil administration

Sildenafil suspension 2.5mg/ml is a compendial product described in the United States Pharmacopeia (USP). It was made by sildenafil citrate tablets (Viagra®, Pfizer) suspended in vehicle, a 1:1 mixture of Ora-sweet and Ora-plus (Paddock Laboratories, Minneapolis, MN). The sildenafil dose was prepared daily by the research pharmacy. If sildenafil was given, the neonate

received sildenafil treatment per os twice daily from day 2 to day 9 of life for a total of 14 doses (2-3mg/kg/dose).

3.3.3 Inflammation-related proteins

On day 2, 3, and 4 of life, blood samples were collected into potassium EDTA coated microtainers from near-term and term neonates with NE (1) without brain injury treated only with TH, (2) with brain injury treated only with TH, and (3) with brain injury treated with TH and sildenafil. Immediately after collection, all the samples were centrifuged at 3600 rpm for 6 minutes at room temperature. Plasma was then extracted, aliquoted, coded and stored at -80°C until tested.

Protein markers were measured blinded to patient identity and disease. The samples were thawed at room temperature, vortexed, and spun at 13,000 x g for 5 minutes for clarification. A 40 mcL of each sample was transferred to a master microtiter plate for multi-analyte profiling (MAP) antigen analysis. An aliquot of each sample was automatically pipetted into one of the capture microsphere multiplexes of the Rules-Based Medicine (Myriad RBM) Custom Human multi-analyte profile (Myriad, Austin, TX) (http://www.rules-basedmedicine.com). These sample and capture microspheres mixtures were thoroughly mixed before incubated at room temperature for 1 hour. Following that, multiplexed cocktails of biotinylated reporter antibodies were added into each multiplex robotically, and were thoroughly mixed prior to incubated at room temperature for another 1 hour. To develop the multiplexes, an excess of streptavidin-phycoerythrin solution was fully mixed into each multiplex and incubated for 1 hour at room temperature. Vacuum filtration was used to reduce the volume of each multiplexed reaction and the volume increased by dilution into matrix buffer for analysis. The multiplexes were analyzed

with a Luminex 100 instrument, and the generated data stream was interpreted using Rules-Based Medicine's (Myriad RBM) proprietary data analysis software. Both calibrators and controls were included on each microtiter plate for each multiplex. The first and last columns of each plate were used to run 8-point calibrator, and 3-level controls were included in duplicate. Testing results for the high, medium and low controls for each multiplex were determined first to guarantee proper assay performance. Unknown values for each analyte that localized in a specific multiplex were acquired utilizing 4- and 5- parameter weighted and non-weighted curve fitting algorithms included in the data analysis package. Expression of 25 inflammation-related proteins was analyzed. Analyzed proteins, listed in **Table 1**, were selected based on inflammatory involvement and assay availability. Unless the sample quantity was not sufficient (QNS), the concentration was below the lower limit of quantification (LLOQ), or the concentration was above the highest quantifiable value, plasma concentrations were reported for each sample.

3.3.4 Baseline plasma concentrations of inflammation-related proteins

On day 2 of life, the blood samples were collected at baseline before sildenafil treatment was started. Plasma concentrations of inflammation-related proteins on DOL 2 were used as baseline levels to assess for initial differences between the groups of neonates.

3.3.5 Data analysis

Kruskal-Wallis tests for continuous variables and Chi-square tests for categorical variables were conducted to compare the differences of clinical characteristics between the three

groups of neonates enrolled. Descriptive data including the mean and standard deviation for continuous variables and the frequency distribution for categorical variables were generated.

The baseline plasma concentrations of inflammatory markers on DOL 2 were assessed for difference between groups using Kruskal-Wallis tests and Dunn's post hoc tests, and their potential impacts on the DOL 3 and DOL 4 protein levels were eliminated in the following statistical analysis. Generalized linear mixed model was used to assess for statistical significance of the differences in protein expressions on DOL 3 and DOL 4, between the three groups of neonates. Descriptive data including the estimated mean and standard deviation/error were generated.

A p value < 0.05 was considered as statistically significant. All statistical analyses were performed with SPSS Version 28.0 for Mac (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 8 (GraphPad Software Inc., San Diego, CA, USA).

3.4 Results

3.4.1 Patients

Twenty-nine neonates with NE treated with TH were included in this study. Five neonates had no brain injury (Group I); 11 neonates who had brain injury were only treated with TH (Group II), and 2 of them died before measurement on DOL 4; and 13 neonates who had brain injury were treated with TH and sildenafil (Group III), and 1 of them died before measurement on DOL 4.

No significant difference in the clinical characteristics was observed between the three groups of neonates (**Table 2**).

3.4.2 Inflammation-related proteins in early days of NE

On day 3 of life, the plasma concentrations of IL-1α were below the LLOQ in 80% (4/5) of Group I neonates, 91% (10/11) of Group II neonates, and 92% (12/13) of Group III neonates; the plasma concentrations of IL-1β were below the LLOQ in all (5/5) neonates from Group I, all (11/11) neonates from Group II, and 92% (12/13) neonates from Group III; the plasma concentrations of IL-12p70 were below the LLOQ in 60% (3/5) neonates from Group I, 54% (6/11) neonates from Group II, and all (13/13) neonates from Group III; the plasma concentrations of IL-17 were below the LLOQ in all (5/5) neonates from Group I, 91% (10/11) neonates from Group II, and 92% (12/13) neonates from Group III. On day 4 of life, the plasma concentrations of IL-1β were below the LLOQ in all (5/5) neonates from Group I, all (9/9) neonates from Group II, and 92% (11/12) neonates from Group III; the plasma concentrations of IL-12p70 were below the LLOQ in 80% (4/5) neonates from Group I, 44% (4/9) neonates from Group II, and all (12/12) neonates from Group III; the plasma concentrations of IL-17 were below the LLOQ in all (5/5) neonates from Group I, all (9/9) neonates from Group II, and 58% (7/12) neonates from Group III. Consequently, the pro-inflammatory cytokines IL-1α, IL-1β, IL-12p70, and IL-17 were excluded from further statistical analysis.

3.4.3 Inflammation-related proteins exhibited different baseline plasma concentrations on day 2 of life between patient groups

Before sildenafil treatment was given on day 2 of life, the plasma concentrations of stem cell factor (SCF) and pulmonary and activation-regulated chemokine (PARC) were significantly higher in neonates with brain injury treated with TH and were going to receive sildenafil (SCF: 646.2 ± 323.7 pg/mL; PARC: 55.92 ± 17.31 ng/mL) than in those with brain injury and only

treated with TH (SCF: 332.0 ± 111.0 pg/mL, p = 0.004; PARC: 20.02 ± 12.08 ng/mL, p < 0.001), and those without brain injury and treated only with TH (SCF: 324.8 ± 78.99 pg/mL, p = 0.01; PARC: 17.10 ± 13.83 ng/mL, p = 0.003) (**Fig. 1A-B**). The plasma concentrations of interleukin-1 receptor antagonist (IL-1Ra), tumor necrosis factor receptor 2 (TNFR2), alpha-2-macroglobulin (A2Macro), beta-2-microglobulin (B2M), vascular cell adhesion molecule-1 (VCAM-1), and tissue inhibitor of metalloproteinases-1 (TIMP-1) were all significantly higher in the TH treated neonates with brain injury who were going to receive sildenafil (IL-1Ra: 620.4 ± 823.4 pg/mL; TNFR2: 42.23 ± 18.89 ng/mL; A2Macro: 3.800 ± 0.451 mg/mL; B2M: 4.400 ± 2.766 μg/mL; VCAM-1: 1820 ± 324.4 ng/mL; TIMP-1: 439.0 ± 336.0 ng/mL), compared to the neonates with brain injury only treated with TH (IL-1Ra: 194.7 ± 89.33 pg/mL, p = 0.03; TNFR2: 19.63 ± 7.739 ng/mL, p = 0.005; A2Macro: 2.209 ± 1.437 mg/mL, p = 0.02; B2M: 2.300 ± 0.504 μg/mL, p = 0.001; VCAM-1: 1328 ± 307.7 ng/mL, p = 0.003; TIMP-1: 187.5 ± 120.6 ng/mL, p = 0.04) (**Fig. 1C-H**).

3.4.4 Brain injury and/or sildenafil increased the levels of inflammatory cytokines

On day 3 of life, there was a significant increase in the plasma concentrations of IL-12p40 in neonates with brain injury treated with TH and sildenafil (0.45 ± 0.05 ng/mL), compared to neonates without brain injury treated only with TH (0.19 ± 0.08 ng/mL) (p = 0.02). While on day 4 of life, no significant difference of IL-12p40 concentrations was observed. (**Fig. 2A**)

On day 4 of life, there was a significant elevation in the plasma concentrations of anti-inflammatory cytokine IL-1Ra in neonates with brain injury treated with TH and sildenafil (1169.84 ± 221.31 pg/mL), in comparison to neonates without brain injury treated only with TH

(269.97 ± 379.21 pg/mL) (p = 0.04). The levels of IL-1Ra on day 3 of life demonstrated no significant difference between groups. (**Fig. 2B**)

3.4.5 Sildenafil modulated the levels of chemokine and inflammation mediator

On day 4 of life, sildenafil treatment significantly increased the plasma concentrations of the chemokine Eotaxin-1 in neonates with NE with brain injury treated with TH and sildeanfil (198.86 ± 29.40 pg/mL), compared to those without brain injury treated only with TH (61.88 ± 26.03 pg/mL) (p = 0.009) and those with brain injury treated only with TH (98.56 ± 22.52 pg/mL) (p = 0.03). (**Fig. 3A**)

On both day 3 and day 4 of life, sildenafil treatment significantly reduced the plasma concentrations of the inflammation modulator A2Macro in neonates with NE with brain injury treated with TH and sildenafil (DOL 3: 2.62 ± 0.12 mg/mL; DOL 4: 2.69 ± 0.12 mg/mL), comparing to neonates with brain injury treated only with TH (DOL 3: 3.14 ± 0.13 mg/mL; DOL 4: 3.18 ± 0.13 mg/mL) (DOL 3: p = 0.006; DOL 4: p = 0.01). On day 3 of life, the plasma concentrations of A2Macro were also significantly lower in neonates with NE with brain injury treated with TH and sildenafil (2.62 ± 0.12 mg/mL), than in those without brain injury treated only with TH (3.11 ± 0.19 mg/mL) (p = 0.04). (**Fig. 3B**)

3.5 Discussion

Currently, as the only standard care for moderate to severe NE, TH has been shown to reduce mortality and developmental disabilities (Edwards et al., 2010; Jacobs et al., 2013; Shankaran, 2012; Wintermark, 2011), yet this reduction is limited around 11%, from 58% to 47% (Edwards et al., 2010; Hassell et al., 2015). In addition, TH solely exerts neuroprotective

effects by preventing the development of brain injury, but fails to restore the damage once it has occurred (Chakkarapani et al., 2021; Gluckman et al., 2005; Shankaran et al., 2005; Yazdani et al., 2016). Moreover, TH does not confer neuroprotection in all cases. Both whole-body cooling and selective head cooling have been shown with less effectiveness in neonates with more severe NE (Azzopardi et al., 2009; Gluckman et al., 2005). A pressing need for complementary treatment to TH is brought to the attention of the public yet not fulfilled. Therefore, we aimed to explore the potential of sildenafil as a promising candidate for adjuvant therapeutic approach.

In pre-clinical studies, sildenafil has been indicated to mediate cerebral blood flow (CBF) redistribution, mitigate hypoxic-ischemic (HI) damage, promote neurogenesis, improve motor locomotion, and reduce apoptosis and inflammation in rat models of NE (Charriaut-Marlangue et al., 2014; Yazdani et al., 2016). An intraperitoneal injection of sildenafil immediately after the initial HI insult in post-natal day 7 (P7) rats inhibited reactive astrogliosis and microglia activation at 72 hours and 7 days after HI (Charriaut-Marlangue et al., 2014). Prior study from our lab in rat model of NE also demonstrated that sildenafil treatment reduced the number of microglia and astrocytes, and modulated the expression of the cytokines including tumor necrosis factor-α (TNFα), IL-1β, and IL-1Ra (Yazdani et al., 2021). However, animal models of NE cannot entirely mimic the human condition (Bracken, 2009), especially when NE has many complex contributing factors at play (Aslam et al., 2019; Gillam-Krakauer & Gowen, 2021). To our knowledge, our study for the first time reported the effects of sildenafil on inflammation in human neonates with NE, representing a significant step in translating the discoveries on sildenafil from bench to bedside.

Three time-points of measurements were included in this study, including DOL 2, 3, and 4. One of the strengths of this research is that the baseline plasma concentrations for each

inflammatory marker before sildenafil treatment was given on DOL 2 were measured and assessed for difference between groups. We observed significantly higher baseline levels of several inflammation-related proteins including SCF, PARC, IL-1Ra, TNFR2, A2Macro, B2M, VCAM-1 and TIMP-1 in the sildenafil-treated group. Therefore, the baseline concentrations for each marker on DOL 2 were adjusted and fixed at an equal level between groups to eliminate their potential impacts from the analysis on DOL 3 and DOL 4. The adjusted analysis on DOL 3 and DOL 4 can better reflect the impact of the sildenafil treatment.

By comparing the neonates with brain injury treated with TH and sildenafil to those without brain injury treated only with TH, we discovered that the brain injury and/or sildenafil upregulated the plasma levels of IL-12p40 and IL-1Ra, on DOL 3 and DOL 4 respectively.

As far as we know, few study has revealed the effect of sildenafil on IL-12p40. One recent clinical study demonstrated that sildenafil administration appeared to reduce the IL-12p40 expression in 21 fertile women (Kniotek et al., 2021). Whereas in our results, IL-12p40 was upregulated in the sildenafil treated brain injured neonates. To be noticed, we were not able to fully discern the impact of brain injury development and the sildenafil treatment when significant difference was observed comparing to neonates who had no brain injury and no sildenafil treatment. IL-12p40 is a subunit of the pro-inflammatory cytokine IL-12, and at the same time may function as an individual cytokine (Cooper & Khader, 2007; Gee et al., 2009; Trinchieri, G., 2003). When act independently, IL-12p40 and its homodimer have been found with both pro- and anti-inflammatory properties. They may chemoattract macrophages via IL-12Rβ1 and result in a pathogenic buildup (Ha et al., 1999; Russell et al., 2003; Walter et al., 2001). On the contrary, they appear to be powerful antagonists against IL-12, competitively binding to the common receptor IL-12Rβ1 and inhibiting the generation of interferon gamma (IFNγ) triggered

by IL-12 (Cooper & Khader, 2007; Gately et al., 1996; Gillessen et al., 1995; Mattner et al., 1993; Vignali & Kuchroo, 2012). Activated microglia were shown to produce IL-12 and IL-12p40 (Aloisi et al., 1997), and the IL-12 signaling in macrophages appeared to be a major pathway activated in human neonates with NE within 12 hours of birth (Montaldo et al., 2019). In a previous clinical study including 13 neonates with NE treated with TH and 39 healthy control neonates, considerably higher serum level of IL-12p40 at birth were found in those with NE, compared to the healthy controls (Go et al., 2021). However, there was no significant difference of the IL-12p40 serum level at birth between 5 neonates with NE treated with TH who had cerebral palsy (CP) and 8 who had no CP (Go et al., 2021). These findings indicated that IL-12p40 was activated early in NE but this initial level did not specifically link to the eventual brain damage development. In a pre-clinical study employing nonhuman primate model of NE, the plasma level of IL-12p40 was significantly higher at 24 and 72 hours of life in the 8 animals survived without CP, comparing to the 14 animals died or survived with CP (Wood et al., 2021). It was suspected that IL-12p40 counterregulated IL-12 and prevented excessive inflammatory responses (Wood et al., 2021). This expression timing of IL-12p40 is in line with our finding that the IL-12p40 levels were upregulated in the sildenafil treated group on day 3 of life (i.e., around 72 hours after birth), and returned to a lower level on DOL 4. The transient upregulation might be beneficial in terms of improving the outcome as indicated in the primate model of NE. Further works to follow up the neurological outcomes in human neonates are required for better understanding of the impacts of sildenafil and IL-12p40.

IL-1Ra is an anti-inflammatory cytokine that belongs to the IL-1 cytokine family and so does the pro-inflammatory cytokine IL-1β. IL-1Ra functions by binding to the IL-1 receptors and antagonizing the pro-inflammatory actions of IL-1β, rather than acting as an functioning agonist

to induce intracellular responses (Arend et al., 1998; Youn et al., 2013). While originally expressed at a very low level in central nervous system (CNS) (Youn et al., 2013), IL-1β has been shown to be vigorously produced in excess following the initial insult of NE (Bona et al., 1999; Deng et al., 2008; Hagberg et al., 1996; Szaflarski et al., 1995). Demonstrated in experimental studies of NE, the activation of IL-1β may induce neurotoxic cascades such as nitric oxide production or excitotoxic damage exacerbation, injure the developing oligodendrocytes, and impair myelination, thus contributing to the brain damage in NE (Cai et al., 2004; Girard et al., 2012; Liu & McCullough, 2013; Savard et al., 2013). In clinical studies, higher levels of IL-1β in biological fluids have also been found associated with higher severity and worse neurological outcomes in neonates with NE (Aly et al., 2006; Bartha et al., 2004; Liu & Feng, 2010; Youn et al., 2012). IL-1Ra itself has also been indicated to be elevated in serum at birth in neonates with NE (Go et al., 2021), corroborating the idea that the activated IL-1β system induces the expression of IL-1Ra following brain injury (Youn et al., 2013). In a study that involved 13 neonates who experienced NE-induced seizures and 15 healthy control neonates, increased serum level of IL-1Ra within the first 24 hours of life was associated with CP at 6 months of age, and decreased level of IL-1Ra later at 48 to 72 hours of life was associated with the occurrence of seizure, suggesting that the lack of consistent induction of IL-1Ra leaves the neonates more vulnerable to seizures (Youn et al., 2012). Hence, not surprisingly, IL-1Ra has been widely studied as a therapeutic option in CNS injuries including stroke, traumatic brain injury (TBI), spinal cord injury (SCI), and NE. In rodent models of stroke, the application of IL-1Ra appears to exert neuroprotection by mitigating leukocytes infiltration, and decreasing the number of necrotic neurons (Garcia et al., 1995; Loddick et al., 1997; Shaftel et al., 2008; Yang et al., 1998). In rat model of TBI, IL-1Ra was able to reduce the brain lesion volume (Toulmond

& Rothwell, 1995). In mouse SCI, peripheral administration of IL-1Ra attenuated the immune cell infiltration at the spinal injury site (Yates et al., 2021). In inflammation-primed rat model of NE, post-natal administration of IL-1Ra appeared to protect myelination and neural stem cell population, decrease long-term gliosis, and improve neurobehavioral outcomes (Girard et al., 2012). In fetal sheep with inflammation-induced NE, IL-1Ra infusion was able to facilitate the recovery of EEG activity and noticeably reduce systemic inflammation, microgliosis, and oligodendrocyte loss (Kelly et al., 2021). These aforementioned findings in together uncontroversially supported that IL-1Ra plays a beneficial role in resolving inflammation and improving brain damage in NE. Sildenafil has displayed its ability in suppressing the expression of IL-1β in various neurological conditions, such as in activated microglial cell culture, multiple sclerosis (MS), and Alzheimer's disease (AD) (Araújo et al., 2020; Nunes et al., 2015; Zhang et al., 2013; Zhao et al., 2011). In our lab's preliminary study using a P7 rat model of NE, sildenafil also appeared to improve the level of IL-1Ra in the white matter. The elevated plasma level of IL-1Ra on DOL 4 in the sildenafil treated group in our study agree with our preliminary discovery in rat model of NE that sildenafil increased the IL-1Ra expression. Our results may serve as evidences for sildenafil's inflammatory resolving effects, and further support the promising therapeutic value of it in NE.

We also found that sildenafil treatment significantly increased the plasma level of eotaxin-1 on DOL 4, and decreased the level of A2Macro on both DOL 3 and 4.

Eotaxin-1 is a well-recognized chemoattractant for leukocytes such as eosinophil, macrophages, and microglia, as well as neural progenitor cells (NPCs) and epithelial cells (Teixeira et al., 2018; Wakabayashi et al., 2021). Apart from promoting the recruitment of microglia, it has also been demonstrated to enhance the subsequent production of reactive

oxygen species (ROS), and the glutamate-mediated neuronal cell deaths (Parajuli et al., 2015). In mouse models of adult stroke, circulating level of eotaxin-1 was shown to be elevated, and a consistent elevation for days was shown to exacerbate acute brain injury and short-term neurological impairment (Lieschke et al., 2019; Roy-O'Reilly et al., 2017). The lack of effects on chronic brain injury by eotaxin-1 was suspected as a consequence of neurorestoration induced by eotaxin-1 (Lieschke et al., 2019). In 133 adult human stroke patients, lower serum level of eotaxin-1 around 24 hours after stroke was found associated with increased severity and poorer functional outcomes at 12 months after stroke, suggesting a protective role of eotaxin-1 following brain injury (Roy-O'Reilly et al., 2017). In adolescent mouse model of stroke, eotaxin-1 was shown to promote neurogenesis and gliogenesis, but not in adult mouse with stroke (Lieschke et al., 2019). In a P9 mouse model of NE, eotaxin-1 was increased at the injury site where NPCs migrated towards, and it was further verified in vitro that eotaxin-1 promoted NPC proliferation and migration, which again implied the neurorestorative effect of eotaxin-1 (Wang et al., 2017). According to the above findings, it is possible that a continuous upregulation of eotaxin-1 following initial insult worsens the acute brain damage and impair short-term neurological outcomes, but the elevation of eotaxin-1, no matter transient or continuous, exerts a neurorestorative effect and results in better long-term prognosis. Sildenafil has been previously indicated to promote neurogenesis in adult stroke (Bednar, 2008; Ding et al., 2008; Li et al., 2007; Zhang et al., 2002) and in rat models of NE (Charriaut-Marlangue et al., 2014; Yazdani et al., 2016). The higher level of eotaxin-1 displayed in sildenafil treated neonates on DOL 4 might be a part of the neurorestorative mechanisms of sildenafil. From our findings, we are not sure whether this elevation is transient or continued. Additional investigations on eotaxin-1 level beyond DOL 4 and on the long-term outcome of these neonates are crucial for future research.

A2Macro is a multifunctional protein, most well-known as a broad-spectrum protease inhibitor (Vandooren & Itoh, 2021). It is recognized to have multiple roles in inflammation, including enhancing leukocytes recruitment, promoting antigen presentation, stimulating production of ROS, and binding a variety of cytokines and growth factors (Canova et al., 2015; Vandooren & Itoh, 2021). Nonetheless, the implications behind many of its functions remain elusive. For instance, the binding of A2Macro to TNFα was suspected to hinder the clearance of TNFα and therefore facilitate the development of lipopolysaccharide (LPS)-induced fever in mice (Gourine et al., 2002). Whereas an in vitro study has demonstrated that A2Macro upon modifications by ROS sequestered pro-inflammatory proteins including TNFα, IL-2, and IL-6, and potentially suppressed the acute phase inflammation (Cater et al., 2019; Wu et al., 1998). A2Macro was also shown in clinical and parallel in vitro studies to be released by perivascular astrocytes and peripheral sources such as liver and blood cells following blood-brain barrier (BBB) disruption (Cucullo et al., 2003). In turn, A2Macro appeared to induce the expression of matrix metalloproteinase-9 (MMP-9) which was discovered to pathologically degrade BBB following ischemic stroke (Turner & Sharp, 2016), in mouse macrophage-derived cell lines (Cáceres et al., 2010). Although some previous studies suggested that A2Macro as a proteinase inhibitor neutralizes MMPs (Beekman et al., 1999; Nagase et al., 1994; Woessner J. F., 1999). These controversial findings indicate that the effects of A2Macro might be context-dependent and require further exploration. In the context of brain injuries, the expression of A2Macro demonstrated an increase following TBI in rat model, potentially due to the activation of signal transducer and activator of transcription-3 (STAT3) pathway (Oliva et al., 2012; Uskoković et al., 2007; Zhang & Darnell, 2001), which has also been indicated in other cerebral insults including stroke, status epilepticus (Raible et al., 2014), and NE (Melo et al., 2021; Zeng et al., 2020). In

agreement with this finding, higher serum level of A2Macro was found in patients with stroke and was associated with more severe white matter lesions in a clinical study with 159 acute ischemic stroke patients and 77 control patients (Nezu et al., 2013). In a P7 mouse model of NE, inhibition of STAT3 resulted in a significant reduction in cell death, tissue loss, microglial and astroglial activation (Hristova et al., 2016). Interestingly, sildenafil appeared to suppress the STAT3 signaling, and reduce the pro-inflammatory cytokines IL-6 and IL-8 induced by ROS in systemic sclerosis fibroblasts (Di Luigi et al., 2020). It is likely that the reduction of A2Macro in our findings was a result of inhibition of STAT3 by sildenafil, and this inhibition may lead to neuroprotection in the context of NE.

Certain limitations exist in this study. First of all are the relatively small group sizes. As a result, more research is required to validate our findings before extrapolating them to a larger population. Secondly, due to the limitation of the measurement method, we were not able to obtain the plasma concentrations for a few interesting markers such as IL-1α, IL-1β, and IL-12p70.

In future research, investigating the evolution over time of plasma inflammatory biomarkers appears an intriguing direction. Since inflammation is an active process: inflammation responders such as immune cells and cytokines are crucial for initial inflammation suppression, yet when the inflammatory conditions remain unresolved, secondary damages and/or persistent cytotoxicity may occur and constantly disrupt the return to homeostasis (Bernis et al., 2022; Fleiss et al., 2021). Inflammatory proteins expressed at different timings may have different functional implications as appeared for IL-12p40 and eotaxin-1. Moreover, further studies on the long-term neurological outcome for the neonates may also help understand the role of inflammatory markers and the impact of sildenafil.

3.6 Conclusion

In conclusion, our findings suggested that sildenafil has potential inflammation-modulating effects by enhancing cytokines with anti-inflammatory activities (IL-12p40 and IL-1Ra) and reducing pro-inflammatory mediator (A2Macro), and potential neurorestorative effect by improving NPCs proliferation (eotaxin-1). The findings are in agreement with results obtained in animal models of NE, and provide first evidence of sildenafil as a promising adjunctive treatment for NE.

3.8 References

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. *Developmental neuroscience*, *39*(1-4), 59–65. https://doi.org/10.1159/000471508

al Naqeeb, N., Edwards, A. D., Cowan, F. M., & Azzopardi, D. (1999). Assessment of neonatal encephalopathy by amplitude-integrated electroencephalography. Pediatrics, 103(6 Pt 1), 1263–1271. https://doi.org/10.1542/peds.103.6.1263

Al-Macki, N., Miller, S. P., Hall, N., & Shevell, M. (2009). The spectrum of abnormal neurologic outcomes subsequent to term intrapartum asphyxia. *Pediatric neurology*, *41*(6), 399–405. https://doi.org/10.1016/j.pediatrneurol.2009.06.001

Aloisi, F., Penna, G., Cerase, J., Menéndez Iglesias, B., & Adorini, L. (1997). IL-12 production by central nervous system microglia is inhibited by astrocytes. *Journal of immunology (Baltimore, Md. : 1950)*, *159*(4), 1604–1612.

Aly, H., Khashaba, M. T., El-Ayouty, M., El-Sayed, O., & Hasanein, B. M. (2006). IL-1β, IL-6 and TNF-α and outcomes of neonatal hypoxic ischemic encephalopathy. *Brain and Development, 28*(3), 178-182. doi:https://doi.org/10.1016/j.braindev.2005.06.006

Araújo, S., Duarte-Silva, E., Marinho, C., Oliveira, W. H., França, M., Lós, D., Peron, G., Tomaz, L., Bonfanti, A. P., Verinaud, L., & Peixoto, C. A. (2020). Effect of sildenafil on neuroinflammation and synaptic plasticity pathways in experimental autoimmune encephalomyelitis. *International immunopharmacology*, *85*, 106581. https://doi.org/10.1016/j.intimp.2020.106581

Arend, W. P., Malyak, M., Guthridge, C. J., & Gabay, C. (1998). Interleukin-1 receptor antagonist: role in biology. *Annual review of immunology*, *16*, 27–55. https://doi.org/10.1146/annurev.immunol.16.1.27

Aslam, S., Strickland, T., & Molloy, E. J. (2019). Neonatal Encephalopathy: Need for Recognition of Multiple Etiologies for Optimal Management. *Frontiers in Pediatrics, 7*.

Azzopardi, D. V., Strohm, B., Edwards, A. D., Dyet, L., Halliday, H. L., Juszczak, E., Kapellou, O., Levene, M., Marlow, N., Porter, E., Thoresen, M., Whitelaw, A., Brocklehurst, P., & TOBY Study Group (2009). Moderate hypothermia to treat perinatal asphyxial encephalopathy. *The New England journal of medicine*, *361*(14), 1349–1358. https://doi.org/10.1056/NEJMoa0900854

Barkovich, A. J., Hajnal, B. L., Vigneron, D., Sola, A., Partridge, J. C., Allen, F., & Ferriero, D. M. (1998). Prediction of neuromotor outcome in perinatal asphyxia: evaluation of MR scoring systems. *AJNR. American journal of neuroradiology*, *19*(1), 143–149.

Bartha, A. I., Foster-Barber, A., Miller, S. P., Vigneron, D. B., Glidden, D. V., Barkovich, A. J., & Ferriero, D. M. (2004). Neonatal Encephalopathy: Association of Cytokines with MR Spectroscopy and Outcome. *Pediatric Research, 56*(6), 960-966. doi:10.1203/01.PDR.0000144819.45689.BB

Bednar M. M. (2008). The role of sildenafil in the treatment of stroke. *Current opinion in investigational drugs (London, England : 2000)*, *9*(7), 754–759.

Beekman, B., Drijfhout, J. W., Ronday, H. K., & TeKoppele, J. M. (1999). Fluorogenic MMP activity assay for plasma including MMPs complexed to alpha 2-macroglobulin. *Annals of the New York Academy of Sciences*, *878*, 150–158. https://doi.org/10.1111/j.1749-6632.1999.tb07681.x

Bernis, M. E., Schleehuber, Y., Zweyer, M., Maes, E., Felderhoff-Müser, U., Picard, D., & Sabir, H. (2022). Temporal Characterization of Microglia-Associated Pro- and Anti-Inflammatory Genes in a Neonatal Inflammation-Sensitized Hypoxic-Ischemic Brain Injury Model. *Oxidative Medicine and Cellular Longevity, 2022*, 2479626. doi:10.1155/2022/2479626

Bona, E., Andersson, A.-L., Blomgren, K., Gilland, E., Puka-Sundvall, M., Gustafson, K., & Hagberg, H. (1999). Chemokine and Inflammatory Cell Response to Hypoxia-Ischemia in Immature Rats. *Pediatric Research, 45*(4), 500-509. doi:10.1203/00006450-199904010-00008

Boudes, E., Tan, X., Saint-Martin, C., Shevell, M., & Wintermark, P. (2015). MRI obtained during versus after hypothermia in asphyxiated newborns. *Archives of Disease in Childhood - Fetal and Neonatal Edition, 100*(3), F238. doi:10.1136/archdischild-2014-306550

Bracken M. B. (2009). Why animal studies are often poor predictors of human reactions to exposure. *Journal of the Royal Society of Medicine*, *102*(3), 120–122. https://doi.org/10.1258/jrsm.2008.08k033

Bryce, J., Boschi-Pinto, C., Shibuya, K., Black, R. E., & WHO Child Health Epidemiology Reference Group (2005). WHO estimates of the causes of death in children. *Lancet (London, England)*, *365*(9465), 1147–1152. https://doi.org/10.1016/S0140-6736(05)71877-8

Cáceres, L. C., Bonacci, G. R., Sánchez, M. C., & Chiabrando, G. A. (2010). Activated α(2) macroglobulin induces matrix metalloproteinase 9 expression by low-density lipoprotein receptor-related protein 1 through MAPK-ERK1/2 and NF-κB activation in macrophage-

derived cell lines. *Journal of cellular biochemistry*, *111*(3), 607–617. https://doi.org/10.1002/jcb.22737

Cai, Z., Lin, S., Pang, Y., & Rhodes, P. G. (2004). Brain injury induced by intracerebral injection of interleukin-1beta and tumor necrosis factor-alpha in the neonatal rat. *Pediatric research*, *56*(3), 377–384. https://doi.org/10.1203/01.PDR.0000134249.92944.14

Canova, D. F., Pavlov, A. M., Norling, L. V., Gobbetti, T., Brunelleschi, S., Le Fauder, P., . . . Perretti, M. (2015). Alpha-2-macroglobulin loaded microcapsules enhance human leukocyte functions and innate immune response. *Journal of Controlled Release, 217*, 284-292. doi:https://doi.org/10.1016/j.jconrel.2015.09.021

Cater, J. H., Wilson, M. R., & Wyatt, A. R. (2019). Alpha-2-Macroglobulin, a Hypochlorite-Regulated Chaperone and Immune System Modulator. *Oxidative medicine and cellular longevity*, *2019*, 5410657. https://doi.org/10.1155/2019/5410657

Chakkarapani, A. A., Aly, H., Benders, M., Cotten, C. M., El-Dib, M., Gressens, P., . . . Robertson, N. J. (2021). Therapies for neonatal encephalopathy: Targeting the latent, secondary and tertiary phases of evolving brain injury. *Seminars in Fetal and Neonatal Medicine, 26*(5), 101256. doi:https://doi.org/10.1016/j.siny.2021.101256

Chalak, L. F., Sánchez, 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. *The Journal of pediatrics*, *164*(3), 468–74.e1. https://doi.org/10.1016/j.jpeds.2013.10.067

Charriaut-Marlangue, C., Nguyen, T., Bonnin, P., Duy, A. P., Leger, P.-L., Csaba, Z., . . . Baud, O. (2014). Sildenafil Mediates Blood-Flow Redistribution and Neuroprotection After

Neonatal Hypoxia-Ischemia. *Stroke, 45*(3), 850-856. doi:10.1161/STROKEAHA.113.003606

Cheong, J. L. Y., Coleman, L., Hunt, R. W., Lee, K. J., Doyle, L. W., Inder, T. E., . . . Infant Cooling Evaluation Collaboration, f. t. (2012). Prognostic Utility of Magnetic Resonance Imaging in Neonatal Hypoxic-Ischemic Encephalopathy: Substudy of a Randomized Trial. *Archives of Pediatrics & Adolescent Medicine, 166*(7), 634-640. doi:10.1001/archpediatrics.2012.284

Cooper, A. M., & Khader, S. A. (2007). IL-12p40: an inherently agonistic cytokine. *Trends in Immunology, 28*(1), 33-38. doi:https://doi.org/10.1016/j.it.2006.11.002

Cucullo, L., Marchi, N., Marroni, M., Fazio, V., Namura, S., & Janigro, D. (2003). Blood-brain barrier damage induces release of alpha2-macroglobulin. *Molecular & cellular proteomics : MCP*, *2*(4), 234–241. https://doi.org/10.1074/mcp.M200077-MCP200

Davidson, J. O., Draghi, V., Whitham, S., Dhillon, S. K., Wassink, G., Bennet, L., & Gunn, A. J. (2018). How long is sufficient for optimal neuroprotection with cerebral cooling after ischemia in fetal sheep? *Journal of Cerebral Blood Flow & Metabolism*, *38*(6), 1047–1059. https://doi.org/10.1177/0271678X17707671

Deng, Y., Lu, J., Sivakumar, V., Ling, E. A., & Kaur, C. (2008). Amoeboid microglia in the periventricular white matter induce oligodendrocyte damage through expression of proinflammatory cytokines via MAP kinase signaling pathway in hypoxic neonatal rats. *Brain pathology (Zurich, Switzerland)*, *18*(3), 387–400. https://doi.org/10.1111/j.1750-3639.2008.00138.x

Di Luigi, L., Sgrò, P., Duranti, G., Sabatini, S., Caporossi, D., Del Galdo, F., Dimauro, I., & Antinozzi, C. (2020). Sildenafil Reduces Expression and Release of IL-6 and IL-8

Induced by Reactive Oxygen Species in Systemic Sclerosis Fibroblasts. *International journal of molecular sciences*, *21*(9), 3161. https://doi.org/10.3390/ijms21093161

Ding, G., Jiang, Q., Li, L., Zhang, L., Zhang, Z. G., Ledbetter, K. A., Panda, S., Davarani, S. P., Athiraman, H., Li, Q., Ewing, J. R., & Chopp, M. (2008). Magnetic resonance imaging investigation of axonal remodeling and angiogenesis after embolic stroke in sildenafil-treated rats. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*, *28*(8), 1440–1448. https://doi.org/10.1038/jcbfm.2008.33

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

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

Fleiss, B., Van Steenwinckel, J., Bokobza, C., K Shearer, I., Ross-Munro, E., & Gressens, P. (2021). Microglia-Mediated Neurodegeneration in Perinatal Brain Injuries. *Biomolecules*, *11*(1), 99. https://doi.org/10.3390/biom11010099

Garcia, J. H., Liu, K. F., & Relton, J. K. (1995). Interleukin-1 receptor antagonist decreases the number of necrotic neurons in rats with middle cerebral artery occlusion. *The American journal of pathology*, *147*(5), 1477–1486.

Gately, M. K., Carvajal, D. M., Connaughton, S. E., Gillessen, S., Warrier, R. R., Kolinsky, K. D., . . . Presky, D. H. (1996). Interleukin-12 Antagonist Activity of Mouse Interleukin-12

p40 Homodimer in Vitro and in Vivo. *Annals of the New York Academy of Sciences, 795*(1), 1-12. doi:https://doi.org/10.1111/j.1749-6632.1996.tb52650.x

Gee, K., Guzzo, C., Che Mat, N. F., Ma, W., & Kumar, A. (2009). The IL-12 family of cytokines in infection, inflammation and autoimmune disorders. *Inflammation & allergy drug targets*, *8*(1), 40–52. https://doi.org/10.2174/187152809787582507

Ghofrani, H. A., Osterloh, I. H., & Grimminger, F. (2006). Sildenafil: from angina to erectile dysfunction to pulmonary hypertension and beyond. *Nature reviews. Drug discovery*, *5*(8), 689–702. https://doi.org/10.1038/nrd2030

Gillam-Krakauer, M., & Gowen Jr, C. W. (2021). Birth Asphyxia. In *StatPearls*. StatPearls Publishing.

Gillessen, S., Carvajal, D., Ling, P., Podlaski, F. J., Stremlo, D. L., Familletti, P. C., . . . Gately, M. K. (1995). Mouse interleukin-12 (IL-12) p40 homodimer: a potent IL-12 antagonist. *European Journal of Immunology, 25*(1), 200-206. doi:https://doi.org/10.1002/eji.1830250133

Girard, S., Sébire, H., Brochu, M. E., Briota, S., Sarret, P., & Sébire, G. (2012). Postnatal administration of IL-1Ra exerts neuroprotective effects following perinatal inflammation and/or hypoxic-ischemic injuries. *Brain, behavior, and immunity*, *26*(8), 1331–1339. https://doi.org/10.1016/j.bbi.2012.09.001

Gluckman, P. D., Wyatt, J. S., Azzopardi, D., Ballard, R., Edwards, A. D., Ferriero, D. M., Polin, R. A., Robertson, C. M., Thoresen, M., Whitelaw, A., & Gunn, A. J. (2005). Selective head cooling with mild systemic hypothermia after neonatal encephalopathy: multicentre randomised trial. *Lancet (London, England)*, *365*(9460), 663–670. https://doi.org/10.1016/S0140-6736(05)17946-X

Go, H., Saito, Y., Maeda, H., Maeda, R., Yaginuma, K., Ogasawara, K., Kashiwabara, N., Kawasaki, Y., & Hosoya, M. (2021). Serum cytokine profiling in neonates with hypoxic ischemic encephalopathy. *Journal of neonatal-perinatal medicine*, *14*(2), 177–182. https://doi.org/10.3233/NPM-200431

Gourine, A. V., Gourine, V. N., Tesfaigzi, Y., Caluwaerts, N., Van Leuven, F., & Kluger, M. J. (2002). Role of α2-macroglobulin in fever and cytokine responses induced by lipopolysaccharide in mice. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology, 283*(1), R218-R226. doi:10.1152/ajpregu.00746.2001

Gunn, A. J., & Gunn, T. R. (1998). The `pharmacology' of neuronal rescue with cerebral hypothermia. *Early Human Development, 53*(1), 19-35. doi:https://doi.org/10.1016/S0378-3782(98)00033-4

Ha, S. J., Lee, C. H., Lee, S. B., Kim, C. M., Jang, K. L., Shin, H. S., & Sung, Y. C. (1999). A Novel Function of IL-12p40 as a Chemotactic Molecule for Macrophages. *The Journal of Immunology, 163*(5), 2902.

Hagberg, H., Gilland, E., Bona, E., Hanson, L. A., Hahin-Zoric, M., Blennow, M., Holst, M., McRae, A., & Söder, O. (1996). Enhanced expression of interleukin (IL)-1 and IL-6 messenger RNA and bioactive protein after hypoxia-ischemia in neonatal rats. *Pediatric research*, *40*(4), 603–609. https://doi.org/10.1203/00006450-199610000-00015

Hassell, K. J., Ezzati, M., Alonso-Alconada, D., Hausenloy, D. J., & Robertson, N. J. (2015). New horizons for newborn brain protection: enhancing endogenous neuroprotection. *Archives of disease in childhood. Fetal and neonatal edition*, *100*(6), F541–F552. https://doi.org/10.1136/archdischild-2014-306284

Hristova, M., Rocha-Ferreira, E., Fontana, X., Thei, L., Buckle, R., Christou, M., Hompoonsup, S., Gostelow, N., Raivich, G., & Peebles, D. (2016). Inhibition of Signal Transducer and Activator of Transcription 3 (STAT3) reduces neonatal hypoxic-ischaemic brain damage. *Journal of neurochemistry*, *136*(5), 981–994. https://doi.org/10.1111/jnc.13490

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. *The Cochrane database of systematic reviews*, *2013*(1), CD003311. https://doi.org/10.1002/14651858.CD003311.pub3

Jenkins, D. D., Rollins, L. G., Perkel, J. K., Wagner, C. L., Katikaneni, L. P., Bass, W. T., Kaufman, D. A., Horgan, M. J., Languani, S., Givelichian, L., Sankaran, K., Yager, J. Y., & Martin, R. H. (2012). Serum cytokines in a clinical trial of hypothermia for neonatal hypoxic-ischemic encephalopathy. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*, *32*(10), 1888–1896. https://doi.org/10.1038/jcbfm.2012.83

Kelly, S. B., Stojanovska, V., Zahra, V. A., Moxham, A., Miller, S. L., Moss, T. J. M., . . . Galinsky, R. (2021). Interleukin-1 blockade attenuates white matter inflammation and oligodendrocyte loss after progressive systemic lipopolysaccharide exposure in near-term fetal sheep. *Journal of Neuroinflammation, 18*(1), 189. doi:10.1186/s12974-021-02238-4

Kniotek, M., Zych, M., Roszczyk, A., Szafarowska, M., & Jerzak, M. M. (2021). Decreased Production of TNF-α and IL-6 Inflammatory Cytokines in Non-Pregnant Idiopathic RPL Women Immunomodulatory Effect of Sildenafil Citrate on the Cellular Response of Idiopathic RPL Women. *Journal of Clinical Medicine*, *10*(14), 3115. https://doi.org/10.3390/jcm10143115

Lee, A. C. C., Kozuki, N., Blencowe, H., Vos, T., Bahalim, A., Darmstadt, G. L., . . . Lawn, J. E. (2013). Intrapartum-related neonatal encephalopathy incidence and impairment at regional and global levels for 2010 with trends from 1990. *Pediatric Research, 74*(1), 50-72. doi:10.1038/pr.2013.206

Li, L., Jiang, Q., Zhang, L., Ding, G., Gang Zhang, Z., Li, Q., . . . Chopp, M. (2007). Angiogenesis and improved cerebral blood flow in the ischemic boundary area detected by MRI after administration of sildenafil to rats with embolic stroke. *Brain Research, 1132*, 185-192. doi:https://doi.org/10.1016/j.brainres.2006.10.098

Lieschke, S., Zechmeister, B., Haupt, M., Zheng, X., Jin, F., Hein, K., Weber, M. S., Hermann, D. M., Bähr, M., Kilic, E., & Doeppner, T. R. (2019). CCL11 Differentially Affects Post-Stroke Brain Injury and Neuroregeneration in Mice Depending on Age. *Cells*, *9*(1), 66. https://doi.org/10.3390/cells9010066

Liu, F., & McCullough, L. D. (2013). Inflammatory responses in hypoxic ischemic encephalopathy. *Acta Pharmacologica Sinica, 34*(9), 1121-1130. doi:10.1038/aps.2013.89

Liu, J., & Feng, Z. C. (2010). Increased umbilical cord plasma interleukin-1 beta levels was correlated with adverse outcomes of neonatal hypoxic-ischemic encephalopathy. *Journal of tropical pediatrics*, *56*(3), 178–182. https://doi.org/10.1093/tropej/fmp098

Loddick, S. A., Wong, M. L., Bongiorno, P. B., Gold, P. W., Licinio, J., & Rothwell, N. J. (1997). Endogenous interleukin-1 receptor antagonist is neuroprotective. *Biochemical and biophysical research communications*, *234*(1), 211–215. https://doi.org/10.1006/bbrc.1997.6436

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. *The Journal of Pediatrics, 194*, 67-75.e61.

Mattner, F., Fischer, S., Guckes, S., Jin, S., Kaulen, H., Schmitt, E., Rüde, E., & Germann, T. (1993). The interleukin-12 subunit p40 specifically inhibits effects of the interleukin-12 heterodimer. *European journal of immunology*, *23*(9), 2202–2208. https://doi.org/10.1002/eji.1830230923

McGowan, M. M., O'Kane, A. C., Vezina, G., Chang, T., Bendush, N., Glass, P., Gai, J., Bost, J., Everett, A. D., & Massaro, A. N. (2021). Serial plasma biomarkers of brain injury in infants with neonatal encephalopathy treated with therapeutic hypothermia. *Pediatric research*, *90*(6), 1228–1234. https://doi.org/10.1038/s41390-021-01405-w

Melo, A. M., Taher, N. A. B., Doherty, D. G., & Molloy, E. J. (2021). The role of lymphocytes in neonatal encephalopathy. *Brain, Behavior, & Immunity - Health, 18*, 100380. doi:https://doi.org/10.1016/j.bbih.2021.100380

Montaldo, P., Kaforou, M., Pollara, G., Hervás-Marín, D., Calabria, I., Panadero, J., Pedrola, L., Lally, P. J., Oliveira, V., Kage, A., Atreja, G., Mendoza, J., Soe, A., Pattnayak, S., Shankaran, S., Vento, M., Herberg, J., & Thayyil, S. (2019). Whole Blood Gene Expression Reveals Specific Transcriptome Changes in Neonatal Encephalopathy. *Neonatology*, *115*(1), 68–76. https://doi.org/10.1159/000492420

Nagase, H., Itoh, Y., & Binner, S. (1994). Interaction of alpha 2-macroglobulin with matrix metalloproteinases and its use for identification of their active forms. *Annals of the New York Academy of Sciences*, *732*, 294–302. https://doi.org/10.1111/j.1749-6632.1994.tb24744.x

Nair, J., & Kumar, V. (2018). Current and Emerging Therapies in the Management of Hypoxic Ischemic Encephalopathy in Neonates. *Children (Basel, Switzerland)*, *5*(7), 99. https://doi.org/10.3390/children5070099

Nezu, T., Hosomi, N., Aoki, S., Deguchi, K., Masugata, H., Ichihara, N., . . . Matsumoto, M. (2013). Alpha2-macroglobulin as a promising biomarker for cerebral small vessel disease in acute ischemic stroke patients. *Journal of Neurology, 260*(10), 2642-2649. doi:10.1007/s00415-013-7040-x

Nunes, A. K. S., Rapôso, C., Rocha, S. W. S., Barbosa, K. P. d. S., de Almeida Luna, R. L., da Cruz-Höfling, M. A., & Peixoto, C. A. (2015). Involvement of AMPK, IKβα-NFκB and eNOS in the sildenafil anti-inflammatory mechanism in a demyelination model. *Brain Research, 1627*, 119-133. doi:https://doi.org/10.1016/j.brainres.2015.09.008

Oliva, A. A., Jr, Kang, Y., Sanchez-Molano, J., Furones, C., & Atkins, C. M. (2012). STAT3 signaling after traumatic brain injury. *Journal of neurochemistry*, *120*(5), 710–720. https://doi.org/10.1111/j.1471-4159.2011.07610.x

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

Osredkar, D., Thoresen, M., Maes, E., Flatebø, T., Elstad, M., & Sabir, H. (2014). Hypothermia is not neuroprotective after infection-sensitized neonatal hypoxic-ischemic brain injury. *Resuscitation*, *85*(4), 567–572. https://doi.org/10.1016/j.resuscitation.2013.12.006

Parajuli, B., Horiuchi, H., Mizuno, T., Takeuchi, H., & Suzumura, A. (2015). CCL11 enhances excitotoxic neuronal death by producing reactive oxygen species in microglia. *Glia*, *63*(12), 2274–2284. https://doi.org/10.1002/glia.22892

Pauliah, S. S., Shankaran, S., Wade, A., Cady, E. B., & Thayyil, S. (2013). Therapeutic hypothermia for neonatal encephalopathy in low- and middle-income countries: a systematic review and meta-analysis. *PloS one*, *8*(3), e58834. https://doi.org/10.1371/journal.pone.0058834

Peixoto, C. A., Nunes, A. K., & Garcia-Osta, A. (2015). Phosphodiesterase-5 Inhibitors: Action on the Signaling Pathways of Neuroinflammation, Neurodegeneration, and Cognition. *Mediators of inflammation*, *2015*, 940207. https://doi.org/10.1155/2015/940207

Raible, D. J., Frey, L. C., & Brooks-Kayal, A. R. (2014). Effects of JAK2-STAT3 signaling after cerebral insults. *JAK-STAT*, *3*(2), e29510. https://doi.org/10.4161/jkst.29510

Roy-O'Reilly, M., Ritzel, R. M., Conway, S. E., Staff, I., Fortunato, G., & McCullough, L. D. (2017). CCL11 (Eotaxin-1) Levels Predict Long-Term Functional Outcomes in Patients Following Ischemic Stroke. *Translational stroke research*, *8*(6), 578–584. https://doi.org/10.1007/s12975-017-0545-3

Russell, T. D., Yan, Q., Fan, G., Khalifah, A. P., Bishop, D. K., Brody, S. L., & Walter, M. J. (2003). IL-12 p40 Homodimer-Dependent Macrophage Chemotaxis and Respiratory Viral Inflammation Are Mediated through IL-12 Receptor β1. *The Journal of Immunology, 171*(12), 6866. doi:10.4049/jimmunol.171.12.6866

Savard, A., Lavoie, K., Brochu, M.-E., Grbic, D., Lepage, M., Gris, D., & Sebire, G. (2013). Involvement of neuronal IL-1β in acquired brain lesions in a rat model of neonatal

encephalopathy. *Journal of Neuroinflammation, 10*(1), 880. doi:10.1186/1742-2094-10-110

Shaftel, S. S., Griffin, W. S. T., & O'Banion, M. K. (2008). The role of interleukin-1 in neuroinflammation and Alzheimer disease: an evolving perspective. *Journal of Neuroinflammation, 5*(1), 7. doi:10.1186/1742-2094-5-7

Shalak, L., & Perlman, J. M. (2004). Hypoxic–ischemic brain injury in the term infant-current concepts. *Early Human Development, 80*(2), 125-141. doi:https://doi.org/10.1016/j.earlhumdev.2004.06.003

Shankaran S. (2012). Therapeutic hypothermia for neonatal encephalopathy. *Current treatment options in neurology*, *14*(6), 608–619. https://doi.org/10.1007/s11940-012-0200-y

Shankaran, S., Laptook, A. R., Ehrenkranz, R. A., Tyson, J. E., McDonald, S. A., Donovan, E. F., Fanaroff, A. A., Poole, W. K., Wright, L. L., Higgins, R. D., Finer, N. N., Carlo, W. A., Duara, S., Oh, W., Cotten, C. M., Stevenson, D. K., Stoll, B. J., Lemons, J. A., Guillet, R., Jobe, A. H., … National Institute of Child Health and Human Development Neonatal Research Network (2005). Whole-body hypothermia for neonates with hypoxic-ischemic encephalopathy. *The New England journal of medicine*, *353*(15), 1574–1584. https://doi.org/10.1056/NEJMcps050929

Silver, B., McCarthy, S., Lu, M., Mitsias, P., Russman, A. N., Katramados, A., . . . Chopp, M. (2009). Sildenafil Treatment of Subacute Ischemic Stroke: A Safety Study at 25-mg Daily for 2 Weeks. *Journal of Stroke and Cerebrovascular Diseases, 18*(5), 381-383. doi:https://doi.org/10.1016/j.jstrokecerebrovasdis.2009.01.007

Simonca, L., & Tulloh, R. (2017). Sildenafil in Infants and Children. *Children (Basel, Switzerland)*, *4*(7), 60. https://doi.org/10.3390/children4070060

Szaflarski, J., Burtrum, D., & Silverstein, F. S. (1995). Cerebral hypoxia-ischemia stimulates cytokine gene expression in perinatal rats. *Stroke*, *26*(6), 1093–1100. https://doi.org/10.1161/01.str.26.6.1093

Teixeira, A. L., Gama, C. S., Rocha, N. P., & Teixeira, M. M. (2018). Revisiting the Role of Eotaxin-1/CCL11 in Psychiatric Disorders. *Frontiers in Psychiatry, 9*.

Toulmond, S., & Rothwell, N. J. (1995). Interleukin-1 receptor antagonist inhibits neuronal damage caused by fluid percussion injury in the rat. *Brain research*, *671*(2), 261–266. https://doi.org/10.1016/0006-8993(94)01343-g

Trinchieri, G. (2003). Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nature Reviews Immunology, 3*(2), 133-146. doi:10.1038/nri1001

Turner, R. J., & Sharp, F. R. (2016). Implications of MMP9 for Blood Brain Barrier Disruption and Hemorrhagic Transformation Following Ischemic Stroke. *Frontiers in cellular neuroscience*, *10*, 56. https://doi.org/10.3389/fncel.2016.00056

Uskoković, A., Dinić, S., Mihailović, M., Grigorov, I., Ivanović-Matić, S., Bogojević, D., Grdović, N., Arambasić, J., Vidaković, M., Martinović, V., Petrović, M., & Poznanović, G. (2007). STAT3/NFkappaB interplay in the regulation of alpha2-macroglobulin gene expression during rat liver development and the acute phase response. *IUBMB life*, *59*(3), 170–178. https://doi.org/10.1080/15216540701272612

Vandooren, J., & Itoh, Y. (2021). Alpha-2-Macroglobulin in Inflammation, Immunity and Infections. *Frontiers in immunology*, *12*, 803244. https://doi.org/10.3389/fimmu.2021.803244

Vignali, D. A. A., & Kuchroo, V. K. (2012). IL-12 family cytokines: immunological playmakers. *Nature Immunology, 13*(8), 722-728. doi:10.1038/ni.2366

Volpe J. J. (2012). Neonatal encephalopathy: an inadequate term for hypoxic-ischemic encephalopathy. *Annals of neurology*, *72*(2), 156–166.

Wakabayashi, K., Isozaki, T., Tsubokura, Y., Fukuse, S., & Kasama, T. (2021). Eotaxin-1/CCL11 is involved in cell migration in rheumatoid arthritis. *Scientific Reports, 11*(1), 7937. doi:10.1038/s41598-021-87199-7

Walsh, B. H., Boylan, G. B., Livingstone, V., Kenny, L. C., Dempsey, E. M., & Murray, D. M. (2013). Cord Blood Proteins and Multichannel-Electroencephalography in Hypoxic-Ischemic Encephalopathy*. *Pediatric Critical Care Medicine, 14*(6).

Walter, M. J., Kajiwara, N., Karanja, P., Castro, M., & Holtzman, M. J. (2001). Interleukin 12 P40 Production by Barrier Epithelial Cells during Airway Inflammation. *Journal of Experimental Medicine, 193*(3), 339-352. doi:10.1084/jem.193.3.339

Wang, F., Baba, N., Shen, Y., Yamashita, T., Tsuru, E., Tsuda, M., . . . Sagara, Y. (2017). CCL11 promotes migration and proliferation of mouse neural progenitor cells. *Stem Cell Research & Therapy, 8*(1), 26. doi:10.1186/s13287-017-0474-9

Wintermark, P. (2011). Current Controversies in Newer Therapies to Treat Birth Asphyxia. *International Journal of Pediatrics, 2011*, 848413. doi:10.1155/2011/848413

Wintermark, P., Boyd, T., Gregas, M. C., Labrecque, M., & Hansen, A. (2010). Placental pathology in asphyxiated newborns meeting the criteria for therapeutic hypothermia. *American journal of obstetrics and gynecology*, *203*(6), 579.e1–579.e5799. https://doi.org/10.1016/j.ajog.2010.08.024

Wintermark, P., Hansen, A., Soul, J., Labrecque, M., Robertson, R. L., & Warfield, S. K. (2011). Early versus late MRI in asphyxiated newborns treated with hypothermia. *Archives of*

disease in childhood. Fetal and neonatal edition, *96*(1), F36–F44. https://doi.org/10.1136/adc.2010.184291

Wisnowski, J. L., Wintermark, P., Bonifacio, S. L., Smyser, C. D., Barkovich, A. J., Edwards, A. D., de Vries, L. S., Inder, T. E., Chau, V., & Newborn Brain Society Guidelines and Publications Committee (2021). Neuroimaging in the term newborn with neonatal encephalopathy. *Seminars in fetal & neonatal medicine*, *26*(5), 101304. https://doi.org/10.1016/j.siny.2021.101304

Woessner J. F., Jr (1999). Matrix metalloproteinase inhibition. From the Jurassic to the third millennium. *Annals of the New York Academy of Sciences*, *878*, 388–403. https://doi.org/10.1111/j.1749-6632.1999.tb07697.x

Wood, T. R., Vu, P. T., Comstock, B. A., Law, J. B., Mayock, D. E., Heagerty, P. J., Burbacher, T., Bammler, T. K., & Juul, S. E. (2021). Cytokine and chemokine responses to injury and treatment in a nonhuman primate model of hypoxic-ischemic encephalopathy treated with hypothermia and erythropoietin. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*, *41*(8), 2054–2066. https://doi.org/10.1177/0271678X21991439

Wu, S. M., Patel, D. D., & Pizzo, S. V. (1998). Oxidized alpha2-macroglobulin (alpha2M) differentially regulates receptor binding by cytokines/growth factors: implications for tissue injury and repair mechanisms in inflammation. *Journal of immunology (Baltimore, Md. : 1950)*, *161*(8), 4356–4365.

Yang, G. Y., Liu, X. H., Kadoya, C., Zhao, Y. J., Mao, Y., Davidson, B. L., & Betz, A. L. (1998). Attenuation of ischemic inflammatory response in mouse brain using an adenoviral vector to induce overexpression of interleukin-1 receptor antagonist. *Journal of cerebral*

blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism, *18*(8), 840–847. https://doi.org/10.1097/00004647-199808000-00004

Yates, A. G., Jogia, T., Gillespie, E. R., Couch, Y., Ruitenberg, M. J., & Anthony, D. C. (2021). Acute IL-1RA treatment suppresses the peripheral and central inflammatory response to spinal cord injury. *Journal of Neuroinflammation, 18*(1), 15. doi:10.1186/s12974-020-02050-6

Yazdani, A., Howidi, B., Shi, M. Z., Tugarinov, N., Khoja, Z., & Wintermark, P. (2021). Sildenafil improves hippocampal brain injuries and restores neuronal development after neonatal hypoxia–ischemia in male rat pups. *Scientific Reports, 11*(1), 22046. doi:10.1038/s41598-021-01097-6

Yazdani, A., Khoja, Z., Johnstone, A., Dale, L., Rampakakis, E., & Wintermark, P. (2016). Sildenafil Improves Brain Injury Recovery following Term Neonatal Hypoxia-Ischemia in Male Rat Pups. *Developmental neuroscience*, *38*(4), 251–263. https://doi.org/10.1159/000448327

Youn, Y. A., Kim, S. J., Sung, I. K., Chung, S. Y., Kim, Y. H., & Lee, I. G. (2012). Serial examination of serum IL-8, IL-10 and IL-1Ra levels is significant in neonatal seizures induced by hypoxic-ischaemic encephalopathy. *Scandinavian journal of immunology*, *76*(3), 286–293. https://doi.org/10.1111/j.1365-3083.2012.02710.x

Youn, Y., Sung, I. K., & Lee, I. G. (2013). The role of cytokines in seizures: interleukin (IL)-1β, IL-1Ra, IL-8, and IL-10. *Korean journal of pediatrics*, *56*(7), 271–274. https://doi.org/10.3345/kjp.2013.56.7.271

Zeng, J., Zhao, Y. L., Deng, B. W., Li, X. Y., Xu, J., Wang, L., & Mu, X. H. (2020). *Zhongguo gu shang = China journal of orthopaedics and traumatology*, *33*(2), 190–194. https://doi.org/10.12200/j.issn.1003-0034.2020.02.020

Zhang, J., Guo, J., Zhao, X., Chen, Z., Wang, G., Liu, A., . . . Wang, C. (2013). Phosphodiesterase-5 inhibitor sildenafil prevents neuroinflammation, lowers beta-amyloid levels and improves cognitive performance in APP/PS1 transgenic mice. *Behavioural Brain Research, 250*, 230-237. doi:https://doi.org/10.1016/j.bbr.2013.05.017

Zhang, R., Wang, Y., Zhang, L., Zhang, Z., Tsang, W., Lu, M., . . . Chopp, M. (2002). Sildenafil (Viagra) Induces Neurogenesis and Promotes Functional Recovery After Stroke in Rats. *Stroke, 33*(11), 2675-2680. doi:10.1161/01.STR.0000034399.95249.59

Zhang, X., & Darnell, J. E., Jr (2001). Functional importance of Stat3 tetramerization in activation of the alpha 2-macroglobulin gene. *The Journal of biological chemistry*, *276*(36), 33576–33581. https://doi.org/10.1074/jbc.M104978200

Zhao, S., Zhang, L., Lian, G., Wang, X., Zhang, H., Yao, X., . . . Wu, C. (2011). Sildenafil attenuates LPS-induced pro-inflammatory responses through down-regulation of intracellular ROS-related MAPK/NF-κB signaling pathways in N9 microglia. *International Immunopharmacology, 11*(4), 468-474. doi:https://doi.org/10.1016/j.intimp.2010.12.017

3.9 Figures

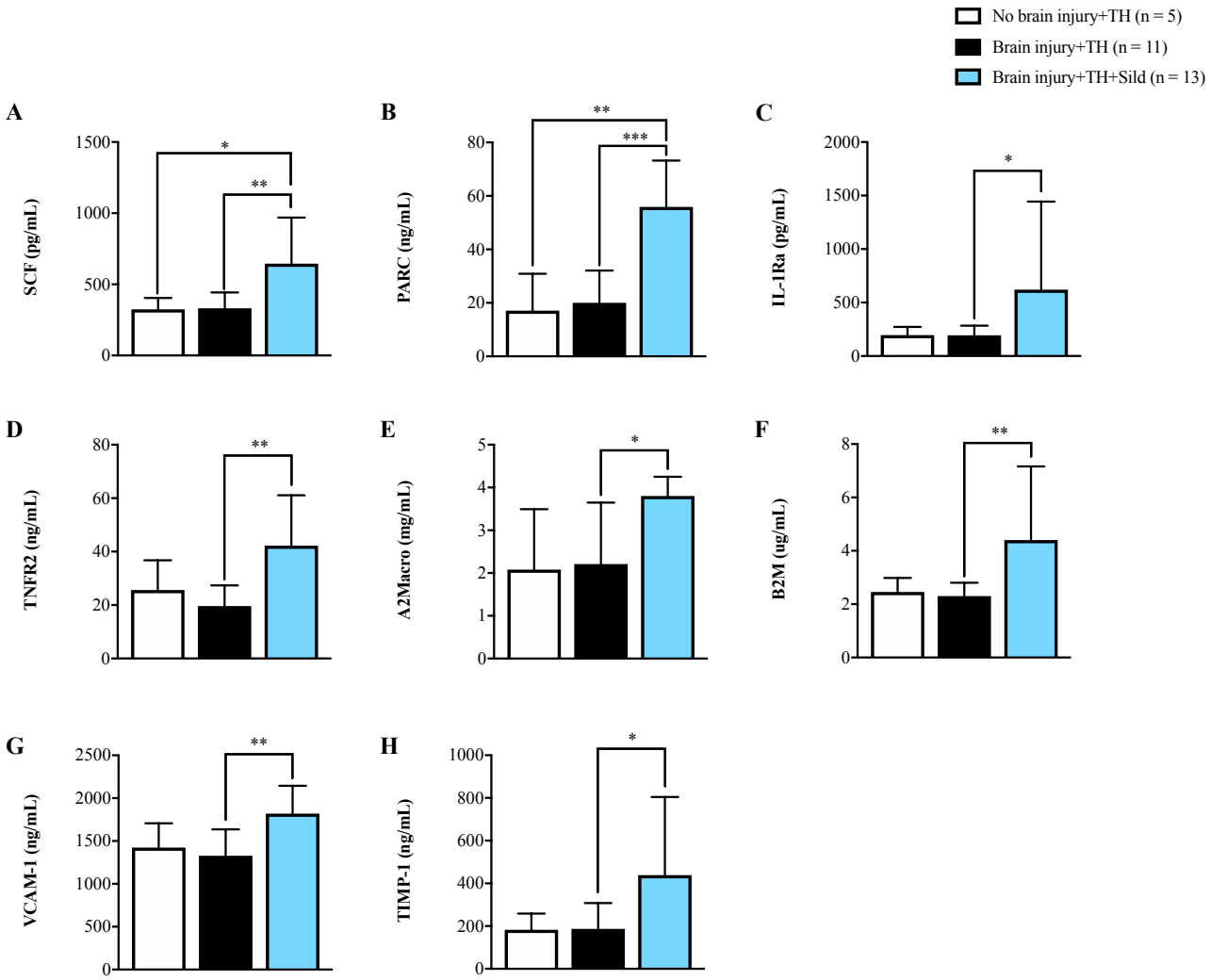

Figure 1. Inflammation-related proteins exhibited different baseline plasma concentrations between groups on day 2 of life. Mean protein concentrations of **(A)** SCF, and **(B)** PARC, (**C**) IL-1Ra, (**D**) TNFR2, (**E**) A2Macro, (**F**) B2M, (**G**) VCAM-1, and (**H**) TIMP-1 were compared between neonates without brain injury treated with TH, neonates with brain injury and only treated with TH, and neonates with brain injury treated with TH and were going to receive sildenafil treatment. DOL, day of life. Mean ± SD. Kruskal-Wallis test and Dunn's post hoc test. Significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

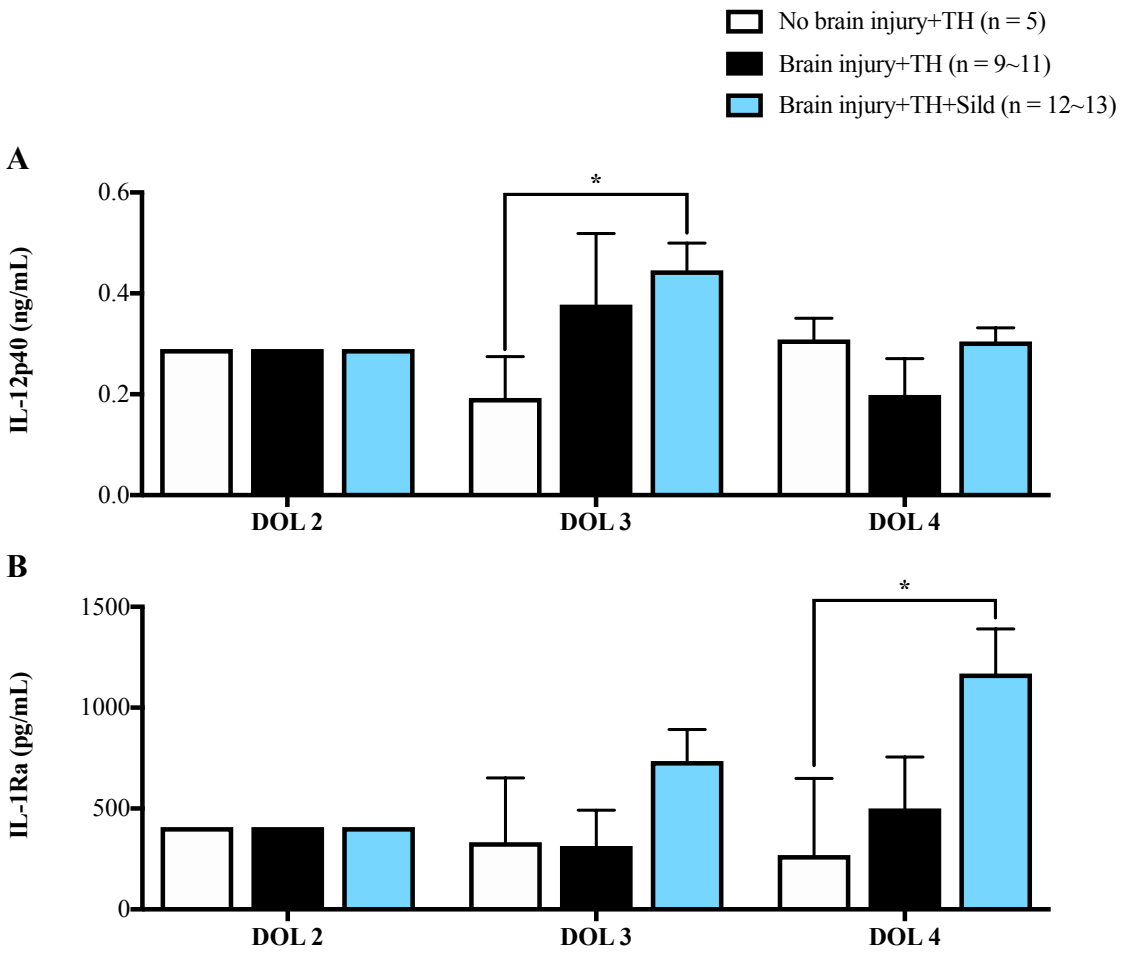

Figure 2. Expression of inflammatory cytokines on day 2, 3, and 4 of life. Mean protein concentrations of **(A)** IL-12p40, and **(B)** IL-1Ra were compared between near-term and term neonates with NE treated with TH (Group I: No brain injury + TH) who did not develop brain injury, (Group II: Brain injury + TH) who developed brain injury and did not receive additional treatment than TH, and (Group III: Brain injury + TH + Sild) who developed brain injury and received sildenafil treatment in addition to TH. The protein concentrations on day of life 2 were fixed at an adjusted level. DOL, day of life. Mean ± SE. Generalized linear mixed model analysis. Significance: * $p < 0.05$.

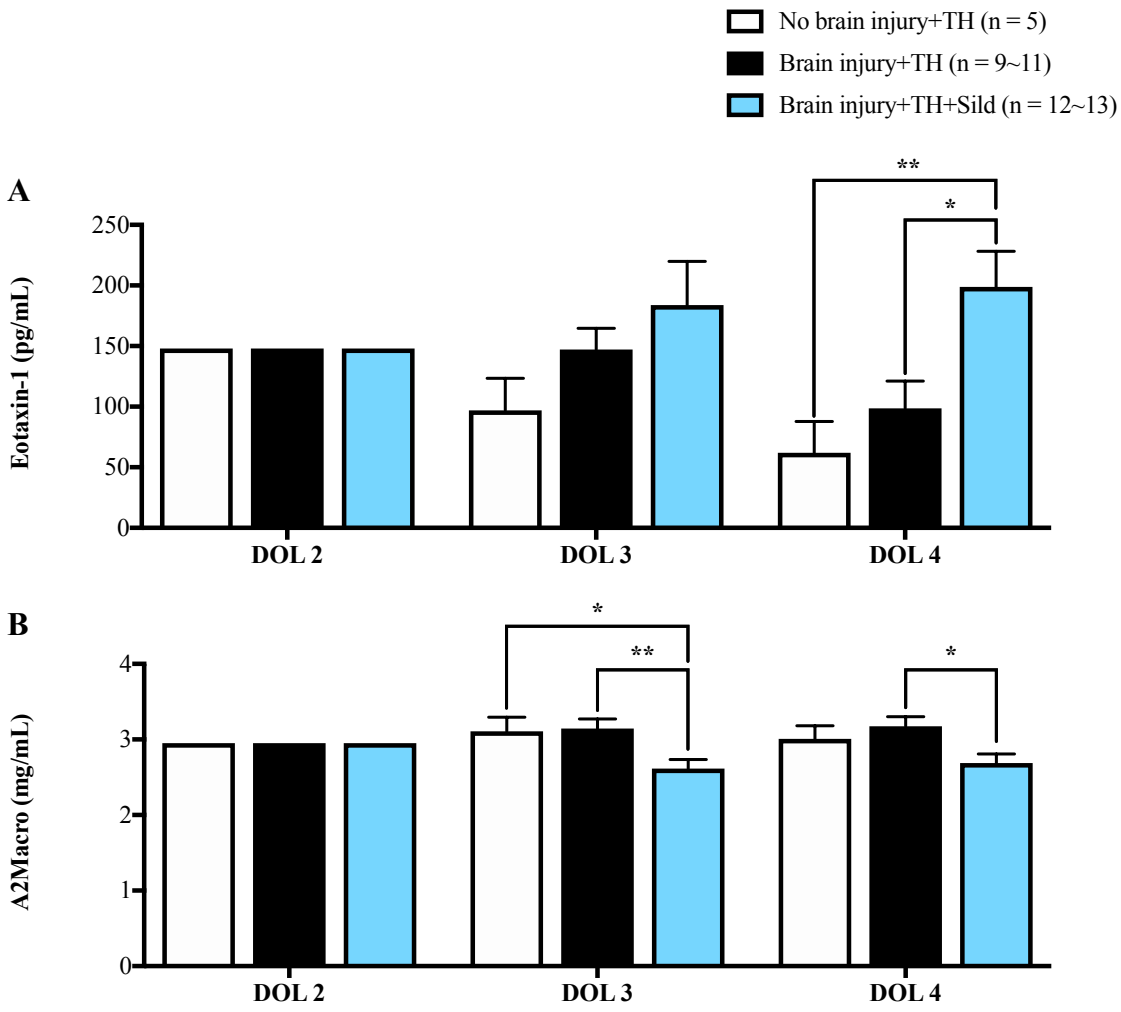

Figure 3. Expression of chemokine and inflammation mediator on day 2, 3, and 4 of life. Mean protein concentrations of **(A)** eotaxin-1, and **(B)** A2Macro were compared between near-term and term neonates with NE treated with TH (Group I: No brain injury + TH) who did not develop brain injury, (Group II: Brain injury + TH) who developed brain injury and did not receive additional treatment than TH, and (Group III: Brain injury + TH + Sild) who developed brain injury and received sildenafil treatment in addition to TH. The protein concentrations on day of life 2 were fixed at an adjusted level. DOL, day of life. Mean ± SE. Generalized linear mixed model analysis. Significance: * $p < 0.05$, ** $p < 0.01$.

3.10 Tables

Table 1. Inflammation-related protein markers analyzed

Inflammation related cytokine	**Chemoattractant**	**Adhesion molecule**
Interleukin-1 alpha (IL-1 alpha)	Beta-2-Microglobulin (B2M)	Intercellular Adhesion Molecule 1 (ICAM-1)
Interleukin-1 beta (IL-1 beta)	Eotaxin-1	Vascular Cell Adhesion Molecule-1 (VCAM-1)
Interleukin-12 Subunit p40 (IL-12p40)	Pulmonary and Activation-Regulated Chemokine (PARC)	
Interleukin-12 Subunit p70 (IL-12p70)	T-Cell-Specific Protein RANTES (RANTES)	**Blood-brain barrier integrity**
Interleukin-17 (IL-17)		Matrix Metalloproteinase-3 (MMP-3)
Stem Cell Factor (SCF)	**Inflammation indicator**	Matrix Metalloproteinase-9 (MMP-9)
	Ferritin (FRTN)	Tissue Inhibitor of Metalloproteinases 1 (TIMP-1)
Anti-inflammation related cytokine	Myoglobin	
Adiponectin		
Interleukin-1 receptor antagonist (IL-1ra)	**Inflammation modulator**	
	Brain-Derived Neurotrophic Factor (BDNF)	
	Factor VII	
Cytokine binding protein/receptor	Plasminogen Activator Inhibitor 1 (PAI-1)	
Alpha-2-Macroglobulin (A2Macro)	Vascular Endothelial Growth Factor (VEGF)	
Tumor necrosis factor receptor 2 (TNFR2)		

The 25 proteins analyzed are categorized based on their predominant role in inflammation. Many of these proteins may be multifunctional and may fit into more than one category.

Table 2. Clinical characteristics of study patients

Genearal characteristic	All neonates with NE treated with TH (n = 29)	Neonates with NE treated with TH without brain injury (n = 5)	Neonates with NE treated with TH with brain injury (n = 11)	Neonates with NE treated with TH with brain injury, and received sildenafil treatment (n = 13)	*P* value
Gestational age (w), mean ± SD	39.50 ± 1.60	38.74 ± 2.02	39.71 ± 1.75	39.61 ± 1.32	*0.53*
Birth weight (g), mean ± SD	3318 ± 651.0	3263 ± 277.3	3206 ± 565.9	3433 ± 818.3	*0.80*
Gender, n (%)					*0.77*
Male	21 (72)	3 (60)	8 (73)	10 (77)	
Female	8 (28)	2 (40)	3 (27)	3 (23)	
Apgar score ≤ 5 at 10 minutes, n (%)	21 (78)	3 (60)	8 (73)	10 (91)	*0.34*
Arterial cord pH, mean ± SD	7.03 ± 0.16	7.08 ± 0.13	6.96 ± 0.16	7.07 ± 0.16	*0.79*
Initial postnatal blood gas pH, mean ± SD	7.05 ± 0.25	7.08 ± 0.12	6.99 ± 0.14	7.09 ± 0.33	*0.51*
Initial modified Sarnat score on admission, n (%)					*0.60*
1	2 (7)	1 (20)	1 (9)	0 (0)	
2	18 (62)	3 (60)	6 (55)	9 (69)	
3	9 (31)	1 (20)	4 (36)	4 (31)	
Initial aEEG background, n (%)					*0.14*
Normal	0 (0)	0 (0)	0 (0)	0 (0)	
Moderately abnormal	13 (45)	4 (80)	3 (27)	6 (46)	
Severely abnormal	16 (55)	1 (20)	8 (73)	7 (54)	
Death, n (%)	4 (14)	0 (0)	2 (18)	2 (15)	*0.60*
BG/W score, n (%)					*0.001*
0	6 (21)	5 (100)	1 (9)	0 (0)	
1	3 (10)	0 (0)	2 (18)	1 (8)	
2	5 (17)	0 (0)	1 (9)	4 (31)	
3	8 (28)	0 (0)	4 (36)	4 (31)	
4	7 (24)	0 (0)	3 (27)	4 (31)	
WBC on DOL2 (10^9/L), mean ± SD	12.17 ± 5.516	9.585 ± 2.120	13.48 ± 4.217	11.84 ± 7.089	*0.40*
Placental pathology, n (%)					*0.41*
Vilitis of unknown etiology	3 (13)	0 (0)	0 (0)	3 (25)	
Chorioamnionitis	10 (40)	1 (33)	5 (50)	3 (25)	
Chorioamnionitis with fetal vasculitis	7 (28)	1 (33)	4 (40)	2 (17)	
Chorioamnionitis without fetal vasculitis	1 (4)	0 (0)	1 (10)	0 (0)	

aEEG, amplitude-integrated electroencephalogram; *BG/W*, Barkovich MRI basal ganglia/watershed score; *DOL*, day of life; *MRI*, magnetic resonance imaging; *NE*, neonatal encephalopathy; *TH*, therapeutic hypothermia; *WBC*, white blood cell count.

aEEG background activity was defined using previously described classifications. (al Naqeeb et al., 1999)

Comprehensive Discussion

Contribution to the thesis hypothesis

In this thesis, we hypothesized that inflammatory biomarkers may provide early guidance for therapeutic interventions in the context of NE, and sildenafil may keep its inflammation-modulating effects in human neonates with NE treated with TH.

In the previous two chapters, we presented the potential plasma inflammatory biomarkers that are associated with the brain injury development in neonates with NE treated with TH, and demonstrated the inflammation-modulating and potential neurorestorative effects of sildenafil as an adjuvant treatment to TH in the early days of NE. In brief, we discussed:

1) In Chapter 2: The plasma levels early on DOL 2 of IL-12p40, SCF, eotaxin-1, and PARC were elevated in neonates with NE treated with TH who developed brain injury, compared to those who had no brain injury. Interestingly, these inflammatory biomarkers exhibited controversial functional implications in the context of brain damage (Doycheva et al., 2013; Go et al., 2021; Kaukola et al., 2004; Keller et al., 2006; Lieschke et al., 2019; Neubauer et al., 2016; Roy-O'Reilly et al., 2017; Wang et al., 2017; Wood et al., 2021; Ziliotto et al., 2018). It is possible that either they were contributing to the brain injury development and TH failed to mitigate their expressions, or they were activated in response to brain injury to exert their protective effects, and in either way they may represent potential therapeutic targets for new adjunctive treatments. Overall, the upregulation of their plasma levels was in association with the diagnosis of brain injury, and therefore these markers may also help distinguish the neonates with NE treated with TH who are in need of additional interventions.

2) In Chapter 3: Brain injury and/or sildenafil appeared to increase the plasma levels of IL-12p40 and IL-1Ra in the early days of NE. Sildenafil was demonstrated to elevate the plasma level of eotaxin-1 and reduce the plasma level of A2Macro in the early days of NE. These expression differences exhibited after sildenafil treatment may reflect the inflammation-modulating (IL-12p40, IL-1Ra, and A2Macro) and potential neurorestorative (eotaxin-1) effects of sildenafil, thus further support its therapeutic value in NE. Our results are in agreement with the previous pre-clinical findings that sildenafil can promote neurogenesis and resolve inflammation in rat models of NE (Charriaut-Marlangue et al., 2014; Yazdani et al., 2016; Yazdani et al., 2021), representing a significant step in translating the discoveries from bench to bedside.

Elevated IL-12p40 and eotaxin-1 by brain injury and sildenafil treatment

With Chapeter 2 as a basis that provided some potential therapeutic targets for adjunctive treatment in NE, we interestingly found that two of them, IL-12p40 and eotaxin-1, appeared to be mediated by sildenafil in early days of NE. The plasma levels of IL-12p40 and eotaxin-1 were upregulated on DOL 2 in neonates who had brain injury, and sildenafil further increased the level of eotaxin-1 on DOL 4 and the level of IL-12p40 transiently on DOL 3. Both IL-12p40 and eotaxin-1 possess pro-inflammatory properties mainly by inducing chemotaxis for leukocytes such as microglia/macrophages and eosinophils (Ha et al., 1999; Parajuli et al., 2015; Russell et al., 2003; Walter et al., 2001; Wakabayashi et al., 2021). The findings that sildenafil did not simply and globally suppress all the inflammatory activities in the early days of NE seem in favor of our hypothesis that sildenafil plays a beneficial role in NE.

It has been recognized in recent years that in the context of brain injury, immune responses are activated to fight off the harmful conditions initially, and when inflammation remains unresolved, the chronic state may result in unfavorable secondary damages and disrupt the return to homeostasis (Bernis et al., 2022; Fleiss et al., 2021; Hagberg et al., 2015). Neuroinflammation is suspected to be not entirely deleterious, and without which the brain damage might be even greater (Ceulemans et al., 2010). This idea is further supported especially since the simply anti-inflammatory strategies applied in early phase of traumatic brain injury (TBI) and stroke did not reach the expected protective effects (Russo & McGavern, 2016; Sughrue et al., 2004). As presented by our results, sildenafil might inhibit excess inflammatory activities by enhancing IL-1Ra and reducing A2Macro, and in the mean while promote eotaxin-1 and IL-12p40 so that the balance is not too tilted. In future research, it is crucial to clarify the effects of neuroinflammatory components at certain timing in the evolving injury progression of NE (Ceulemans et al., 2010; Murray, D. M., 2019), and to study the longer-term neurological outcome, so that we can better understand the role of sildenafil.

Clinical significance

Blood inflammatory biomarkers have advantages such as being objective, serial, and non-invasive (Dietrick et al., 2020), and have been found to change rapidly over time in parallel with the evolving pathophysiological changes in NE (Murray, D. M., 2019), making them potential real-time indicators of the injury progression. Only in recent years, investigations on blood inflammatory biomarkers for their association with the diagnosis, severity, and/or outcome in neonates with NE who received TH treatment have started (McGowan et al., 2021). Our findings in Chapter 2 expand our current knowledge pertaining to the profile of blood inflammatory

biomarkers in neonates with NE who may not respond to TH adequately. The four markers we identified (IL-12p40, SCF, eotaxin-1, and PARC) have potential clinical value to provide guidance for additional therapeutic interventions in NE.

In the study design in Chapter 2, we measured the plasma concentrations of inflammatory biomarkers on DOL 2 before the administration of sildenafil, rather than at an earlier timing. In Chapter 3, we administered the sildenafil treatment starting from DOL 2 for a consecutive of 7 days. This timeline of drug administration was applied in order to match our design in our pre-clinical study using a P10 rat model of NE, in which we induced the HI insult on P10 and started sildenafil treatment on P11 for a consecutive of 7 days (Yazdani et al., 2016; Yazdani et al., 2021). We demonstrated in the rat model of NE that a late start of sildenafil treatment still allows its neurorestorative and inflammation-modulating effects (Yazdani et al., 2016; Yazdani et al., 2021), unlike TH which has a relatively strict therapeutic window (i.e., within 6 hours after birth) and the initiation timing would affect its efficacy (Davidson et al., 2018; Gunn, A. J., & Gunn, T. R., 1998). Therefore, we were intrigued to see whether sildenafil keeps its beneficial effects in a similar design in human neonates, and the inflammatory biomarker measurements on DOL 2 were investigated to see whether they can serve as guidance for the need of sildenafil treatment. Our results supported the viability of conducting these procedures as late as on the second day of life, providing the ill neonates with a longer window for prognostication and starting adjunctive treatment.

Limitations

Although blood biomarkers are under active investigations and are accumulating evidences for their practical values (Ahearne et al., 2017; Bartha et al., 2004; Chalak et al., 2014;

McGowan et al., 2021; Sävman et al., 1998; Walsh et al., 2013; Vasiljevic et al., 2011), none of such biomarkers found associated with the severity and/or outcome of NE is in clinical use presently, potentially due to a lack of enough validation studies to warrant their application into clinical practice (Dietrick et al., 2020; Ramaswamy et al., 2009). In Chapter 2, we identified four markers IL-12p40, SCF, eotaxin-1, and PARC: only IL-12p40 has previous clinical study suggesting its association with NE (Go et al., 2021); PARC exhibited lower expression level at birth that was associated with CP development in previous clinical investigation (Kaukola et al., 2004); as far as we know, eotaxin-1 and SCF had no prior clinical findings that propose their role in NE. Further validations of these markers are required.

The other limitation is that the group size of the neonates with NE treated with TH who did not develop brain injury in both Chapter 2 and Chapter 3 was very limited. Hence additional validations would be necessary before generalizing our results to larger population.

Future directions

This thesis study presented the effects of sildenafil as an adjunctive treatment to TH in human neonates, while in the preliminary results from our lab, we studied the effects of sildenafil alone in rat model of NE without TH treatment (Yazdani et al., 2016; Yazdani et al., 2021). For future research, we have initiated pre-clinical study that includes TH treatment in the study design, which may allow us to better understand the synergistic effect of sildenafil and TH. Moreover, per-clinical animal study may also enable us to carry out a more oriented and more comprehensive investigation on the effects of neuroinflammatory components at different timings in the evolving injury progression of NE, which are crucial for understanding the role of sildenafil.

Clinical study that adds more time points for the measurements of blood inflammatory biomarkers may better provide a dynamic profile of these markers and enhance their predictive power for the brain injury. Studying the longer-term outcomes of the neonates treated with sildenafil to examine whether sildenafil exerts notable benefits in long-term appears to be another important direction for future research.

Final Conclusion

In conclusion, this thesis study first identified the potential involvement of inflammatory biomarkers IL-12p40, SCF, eotaxin-1, and PARC in the development process of brain injury in neonates with NE treated with TH. These inflammatory biomarkers may help assess the risk for brain injury in neonates who received TH treatment, and may also represent potential therapeutic targets for adjunctive therapies. Then we demonstrated sildenafil's regulative role on two of these markers, IL-12p40 and eotaxin-1, and in addition another two inflammatory markers, IL-1Ra and A2Macro. Sildenafil has potential inflammation-modulating effects by enhancing cytokines with anti-inflammatory activities (IL-12p40 and IL-1Ra) and reducing pro-inflammatory mediator (A2Macro), and potential neurorestorative effect by improving NPCs proliferation (eotaxin-1) in early days of NE. The findings agreed with the discoveries in animal models of NE, and further evidenced sildenafil as a promising adjunctive treatment for NE.

Bibliography

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. *Developmental neuroscience*, *39*(1-4), 59–65. https://doi.org/10.1159/000471508

Al-Macki, N., Miller, S. P., Hall, N., & Shevell, M. (2009). The spectrum of abnormal neurologic outcomes subsequent to term intrapartum asphyxia. *Pediatric neurology*, *41*(6), 399–405. https://doi.org/10.1016/j.pediatrneurol.2009.06.001

Allen, K. A., & Brandon, D. H. (2011). Hypoxic Ischemic Encephalopathy: Pathophysiology and Experimental Treatments. *Newborn and infant nursing reviews : NAINR*, *11*(3), 125–133. https://doi.org/10.1053/j.nainr.2011.07.004

Alvarez-Díaz, A., Hilario, E., de Cerio, F. G., Valls-i-Soler, A., & Alvarez-Díaz, F. J. (2007). Hypoxic-ischemic injury in the immature brain--key vascular and cellular players. *Neonatology*, *92*(4), 227–235. https://doi.org/10.1159/000103741

Aoki, Y., Kono, T., Enokizono, M., & Okazaki, K. (2021). Short-term outcomes in infants with mild neonatal encephalopathy: a retrospective, observational study. *BMC Pediatrics, 21*(1), 224. doi:10.1186/s12887-021-02688-y

Araújo, S., Duarte-Silva, E., Marinho, C., Oliveira, W. H., França, M., Lós, D., Peron, G., Tomaz, L., Bonfanti, A. P., Verinaud, L., & Peixoto, C. A. (2020). Effect of sildenafil on neuroinflammation and synaptic plasticity pathways in experimental autoimmune encephalomyelitis. *International immunopharmacology*, *85*, 106581. https://doi.org/10.1016/j.intimp.2020.106581

Aslam, S., Strickland, T., & Molloy, E. J. (2019). Neonatal Encephalopathy: Need for Recognition of Multiple Etiologies for Optimal Management. *Frontiers in Pediatrics, 7*.

Azzopardi, D. V., Strohm, B., Edwards, A. D., Dyet, L., Halliday, H. L., Juszczak, E., Kapellou, O., Levene, M., Marlow, N., Porter, E., Thoresen, M., Whitelaw, A., Brocklehurst, P., & TOBY Study Group (2009). Moderate hypothermia to treat perinatal asphyxial encephalopathy. *The New England journal of medicine*, *361*(14), 1349–1358. https://doi.org/10.1056/NEJMoa0900854

Barkovich, A. J., Hajnal, B. L., Vigneron, D., Sola, A., Partridge, J. C., Allen, F., & Ferriero, D. M. (1998). Prediction of neuromotor outcome in perinatal asphyxia: evaluation of MR scoring systems. *AJNR. American journal of neuroradiology*, *19*(1), 143–149.

Bartha, A. I., Foster-Barber, A., Miller, S. P., Vigneron, D. B., Glidden, D. V., Barkovich, A. J., & Ferriero, D. M. (2004). Neonatal Encephalopathy: Association of Cytokines with MR Spectroscopy and Outcome. *Pediatric Research, 56*(6), 960-966. doi:10.1203/01.PDR.0000144819.45689.BB

Bayley N. (2006) Bayley Scales of Infant and Toddler Development. 3rd edn. *San Antonio, TX: Harcourt Assessment Inc.*

Bednar M. M. (2008). The role of sildenafil in the treatment of stroke. *Current opinion in investigational drugs (London, England : 2000)*, *9*(7), 754–759.

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. *The Journal of physiology*, *578*(Pt 2), 491–506. https://doi.org/10.1113/jphysiol.2006.119602

Bernis, M. E., Schleehuber, Y., Zweyer, M., Maes, E., Felderhoff-Müser, U., Picard, D., & Sabir, H. (2022). Temporal Characterization of Microglia-Associated Pro- and Anti-Inflammatory Genes in a Neonatal Inflammation-Sensitized Hypoxic-Ischemic Brain Injury Model. *Oxidative Medicine and Cellular Longevity, 2022*, 2479626. doi:10.1155/2022/2479626

Bona, E., Andersson, A.-L., Blomgren, K., Gilland, E., Puka-Sundvall, M., Gustafson, K., & Hagberg, H. (1999). Chemokine and Inflammatory Cell Response to Hypoxia-Ischemia in Immature Rats. *Pediatric Research, 45*(4), 500-509. doi:10.1203/00006450-199904010-00008

Bonestroo, H. J. C., Nijboer, C. H. A., van Velthoven, C. T. J., Kavelaars, A., Hack, C. E., van Bel, F., & Heijnen, C. J. (2013). Cerebral and Hepatic Inflammatory Response after Neonatal Hypoxia-Ischemia in Newborn Rats. *Developmental Neuroscience, 35*(2-3), 197-211. doi:10.1159/000346685

Boudes, E., Tan, X., Saint-Martin, C., Shevell, M., & Wintermark, P. (2015). MRI obtained during versus after hypothermia in asphyxiated newborns. *Archives of Disease in Childhood - Fetal and Neonatal Edition, 100*(3), F238. doi:10.1136/archdischild-2014-306550

Bracken M. B. (2009). Why animal studies are often poor predictors of human reactions to exposure. *Journal of the Royal Society of Medicine*, *102*(3), 120–122. https://doi.org/10.1258/jrsm.2008.08k033

Bryce, J., Boschi-Pinto, C., Shibuya, K., Black, R. E., & WHO Child Health Epidemiology Reference Group (2005). WHO estimates of the causes of death in children. *Lancet*

(London, England), *365*(9465), 1147–1152. https://doi.org/10.1016/S0140-6736(05)71877-8

Cardinali D. P. (2019). An Assessment of Melatonin's Therapeutic Value in the Hypoxic-Ischemic Encephalopathy of the Newborn. *Frontiers in synaptic neuroscience*, *11*, 34. https://doi.org/10.3389/fnsyn.2019.00034

Ceulemans, A. G., Zgavc, T., Kooijman, R., Hachimi-Idrissi, S., Sarre, S., & Michotte, Y. (2010). The dual role of the neuroinflammatory response after ischemic stroke: modulatory effects of hypothermia. *Journal of neuroinflammation*, *7*, 74. https://doi.org/10.1186/1742-2094-7-74

Chakkarapani, A. A., Aly, H., Benders, M., Cotten, C. M., El-Dib, M., Gressens, P., . . . Robertson, N. J. (2021). Therapies for neonatal encephalopathy: Targeting the latent, secondary and tertiary phases of evolving brain injury. *Seminars in Fetal and Neonatal Medicine, 26*(5), 101256. doi:https://doi.org/10.1016/j.siny.2021.101256

Chalak, L. F., DuPont, T. L., Sánchez, P. J., Lucke, A., Heyne, R. J., Morriss, M. C., & Rollins, N. K. (2014). Neurodevelopmental outcomes after hypothermia therapy in the era of Bayley-III. Journal of perinatology : official journal of the California Perinatal Association, 34(8), 629–633. https://doi.org/10.1038/jp.2014.67

Chalak, L. F., Sánchez, 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. *The Journal of pediatrics*, *164*(3), 468–74.e1. https://doi.org/10.1016/j.jpeds.2013.10.067

Charriaut-Marlangue, C., Nguyen, T., Bonnin, P., Duy, A. P., Leger, P.-L., Csaba, Z., . . . Baud, O. (2014). Sildenafil Mediates Blood-Flow Redistribution and Neuroprotection After

Neonatal Hypoxia-Ischemia. *Stroke, 45*(3), 850-856. doi:10.1161/STROKEAHA.113.003606

Cheong, J. L. Y., Coleman, L., Hunt, R. W., Lee, K. J., Doyle, L. W., Inder, T. E., . . . Infant Cooling Evaluation Collaboration, f. t. (2012). Prognostic Utility of Magnetic Resonance Imaging in Neonatal Hypoxic-Ischemic Encephalopathy: Substudy of a Randomized Trial. *Archives of Pediatrics & Adolescent Medicine, 166*(7), 634-640. doi:10.1001/archpediatrics.2012.284

Cotten, C. M., & Shankaran, S. (2010). Hypothermia for hypoxic-ischemic encephalopathy. *Expert review of obstetrics & gynecology*, *5*(2), 227–239. https://doi.org/10.1586/eog.10.7

Dammann O. (2007). Persistent neuro-inflammation in cerebral palsy: a therapeutic window of opportunity?. *Acta paediatrica (Oslo, Norway : 1992)*, *96*(1), 6–7. https://doi.org/10.1111/j.1651-2227.2007.00097.x

Davidson, J. O., Draghi, V., Whitham, S., Dhillon, S. K., Wassink, G., Bennet, L., & Gunn, A. J. (2018). How long is sufficient for optimal neuroprotection with cerebral cooling after ischemia in fetal sheep? *Journal of Cerebral Blood Flow & Metabolism*, *38*(6), 1047–1059. https://doi.org/10.1177/0271678X17707671

Davidson, J. O., Gonzalez, F., Gressens, P., & Gunn, A. J. (2021). Update on mechanisms of the pathophysiology of neonatal encephalopathy. *Seminars in Fetal and Neonatal Medicine, 26*(5), 101267. doi:https://doi.org/10.1016/j.siny.2021.101267

Deng, Y., Lu, J., Sivakumar, V., Ling, E. A., & Kaur, C. (2008). Amoeboid microglia in the periventricular white matter induce oligodendrocyte damage through expression of proinflammatory cytokines via MAP kinase signaling pathway in hypoxic neonatal

rats. *Brain pathology (Zurich, Switzerland)*, *18*(3), 387–400. https://doi.org/10.1111/j.1750-3639.2008.00138.x

Dietrick, B., Molloy, E., Massaro, A. N., Strickland, T., Zhu, J., Slevin, M., . . . Everett, A. D. (2020). Plasma and Cerebrospinal Fluid Candidate Biomarkers of Neonatal Encephalopathy Severity and Neurodevelopmental Outcomes. *The Journal of Pediatrics, 226*, 71-79.e75. doi:https://doi.org/10.1016/j.jpeds.2020.06.078

Ding, G., Jiang, Q., Li, L., Zhang, L., Zhang, Z. G., Ledbetter, K. A., Panda, S., Davarani, S. P., Athiraman, H., Li, Q., Ewing, J. R., & Chopp, M. (2008). Magnetic resonance imaging investigation of axonal remodeling and angiogenesis after embolic stroke in sildenafil-treated rats. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*, *28*(8), 1440–1448. https://doi.org/10.1038/jcbfm.2008.33

Doycheva, D., Shih, G., Chen, H., Applegate, R., Zhang, J. H., & Tang, J. (2013). Granulocyte-colony stimulating factor in combination with stem cell factor confers greater neuroprotection after hypoxic-ischemic brain damage in the neonatal rats than a solitary treatment. *Translational stroke research*, *4*(2), 171–178. https://doi.org/10.1007/s12975-012-0225-2

Drury, P. P., Bennet, L., & Gunn, A. J. (2010). Mechanisms of hypothermic neuroprotection. *Seminars in Fetal and Neonatal Medicine, 15*(5), 287-292. doi:https://doi.org/10.1016/j.siny.2010.05.005

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

Favrais, G., van de Looij, Y., Fleiss, B., Ramanantsoa, N., Bonnin, P., Stoltenburg-Didinger, G., Lacaud, A., Saliba, E., Dammann, O., Gallego, J., Sizonenko, S., Hagberg, H., Lelièvre, V., & Gressens, P. (2011). Systemic inflammation disrupts the developmental program of white matter. *Annals of neurology*, *70*(4), 550–565. https://doi.org/10.1002/ana.22489

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

Fleiss, B., Van Steenwinckel, J., Bokobza, C., K Shearer, I., Ross-Munro, E., & Gressens, P. (2021). Microglia-Mediated Neurodegeneration in Perinatal Brain Injuries. *Biomolecules*, *11*(1), 99. https://doi.org/10.3390/biom11010099

Frankenburg, W. K., & Dodds, J. B. (1967). The Denver Developmental Screening Test. *The Journal of Pediatrics, 71*(2), 181-191. doi:https://doi.org/10.1016/S0022-3476(67)80070-2

Fujimoto, T., Sakoda, S., Fujimura, H., & Yanagihara, T. (1999). Ibudilast, a phosphodiesterase inhibitor, ameliorates experimental autoimmune encephalomyelitis in Dark August rats. *Journal of Neuroimmunology, 95*(1), 35-42. doi:https://doi.org/10.1016/S0165-5728(98)00251-3

Ghofrani, H. A., Osterloh, I. H., & Grimminger, F. (2006). Sildenafil: from angina to erectile dysfunction to pulmonary hypertension and beyond. *Nature reviews. Drug discovery*, *5*(8), 689–702. https://doi.org/10.1038/nrd2030

Gillam-Krakauer, M., & Gowen Jr, C. W. (2021). Birth Asphyxia. In *StatPearls*. StatPearls Publishing.

Gluckman, P. D., Wyatt, J. S., Azzopardi, D., Ballard, R., Edwards, A. D., Ferriero, D. M., Polin, R. A., Robertson, C. M., Thoresen, M., Whitelaw, A., & Gunn, A. J. (2005). Selective head cooling with mild systemic hypothermia after neonatal encephalopathy: multicentre randomised trial. *Lancet (London, England)*, *365*(9460), 663–670. https://doi.org/10.1016/S0140-6736(05)17946-X

Go, H., Saito, Y., Maeda, H., Maeda, R., Yaginuma, K., Ogasawara, K., Kashiwabara, N., Kawasaki, Y., & Hosoya, M. (2021). Serum cytokine profiling in neonates with hypoxic ischemic encephalopathy. *Journal of neonatal-perinatal medicine*, *14*(2), 177–182. https://doi.org/10.3233/NPM-200431

Greco, P., Nencini, G., Piva, I., Scioscia, M., Volta, C. A., Spadaro, S., . . . Nappi, L. (2020). Pathophysiology of hypoxic–ischemic encephalopathy: a review of the past and a view on the future. *Acta Neurologica Belgica, 120*(2), 277-288. doi:10.1007/s13760-020-01308-3

Grow, J., & Barks, J. D. E. (2002). Pathogenesis of hypoxic-ischemic cerebral injury in the term infant: current concepts. *Clinics in Perinatology, 29*(4), 585-602. doi:https://doi.org/10.1016/S0095-5108(02)00059-3

Gunn, A. J., & Gunn, T. R. (1998). The `pharmacology' of neuronal rescue with cerebral hypothermia. *Early Human Development, 53*(1), 19-35. doi:https://doi.org/10.1016/S0378-3782(98)00033-4

Ha, S. J., Lee, C. H., Lee, S. B., Kim, C. M., Jang, K. L., Shin, H. S., & Sung, Y. C. (1999). A Novel Function of IL-12p40 as a Chemotactic Molecule for Macrophages. *The Journal of Immunology, 163*(5), 2902.

Hagberg, H., Gilland, E., Bona, E., Hanson, L. A., Hahin-Zoric, M., Blennow, M., Holst, M., McRae, A., & Söder, O. (1996). Enhanced expression of interleukin (IL)-1 and IL-6 messenger RNA and bioactive protein after hypoxia-ischemia in neonatal rats. *Pediatric research*, *40*(4), 603–609. https://doi.org/10.1203/00006450-199610000-00015

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. *Nature reviews. Neurology*, *11*(4), 192–208. https://doi.org/10.1038/nrneurol.2015.13

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

Hanrahan, J. D., Sargentoni, J., Azzopardi, D., Manji, K., Cowan, F. M., Rutherford, M. A., Cox, I. J., Bell, J. D., Bryant, D. J., & Edwards, A. D. (1996). Cerebral metabolism within 18 hours of birth asphyxia: a proton magnetic resonance spectroscopy study. *Pediatric research*, *39*(4 Pt 1), 584–590. https://doi.org/10.1203/00006450-199604000-00004

Hassell, K. J., Ezzati, M., Alonso-Alconada, D., Hausenloy, D. J., & Robertson, N. J. (2015). New horizons for newborn brain protection: enhancing endogenous neuroprotection. *Archives of disease in childhood. Fetal and neonatal edition*, *100*(6), F541–F552. https://doi.org/10.1136/archdischild-2014-306284

He, M. L., Lv, Z. Y., Shi, X., Yang, T., Zhang, Y., Li, T. Y., & Chen, J. (2017). Interleukin-10 release from astrocytes suppresses neuronal apoptosis via the TLR2/NFκB pathway in a

neonatal rat model of hypoxic-ischemic brain damage. *Journal of neurochemistry*, *142*(6), 920–933. https://doi.org/10.1111/jnc.14126

Hedtjärn, M., Leverin, A. L., Eriksson, K., Blomgren, K., Mallard, C., & Hagberg, H. (2002). Interleukin-18 involvement in hypoxic-ischemic brain injury. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *22*(14), 5910–5919. https://doi.org/10.1523/JNEUROSCI.22-14-05910.2002

Hellström Erkenstam, N., Smith, P. L. P., Fleiss, B., Nair, S., Svedin, P., Wang, W., . . . Mallard, C. (2016). Temporal Characterization of Microglia/Macrophage Phenotypes in a Mouse Model of Neonatal Hypoxic-Ischemic Brain Injury. *Frontiers in Cellular Neuroscience, 10*.

Hudome, S., Palmer, C., Roberts, R. L., Mauger, D., Housman, C., & Towfighi, J. (1997). The Role of Neutrophils in the Production of Hypoxic-Ischemic Brain Injury in the Neonatal Rat. *Pediatric Research, 41*(5), 607-616. doi:10.1203/00006450-199705000-00002

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. *The Cochrane database of systematic reviews*, *2013*(1), CD003311. https://doi.org/10.1002/14651858.CD003311.pub3

Jenkins, D. D., Rollins, L. G., Perkel, J. K., Wagner, C. L., Katikaneni, L. P., Bass, W. T., Kaufman, D. A., Horgan, M. J., Languani, S., Givelichian, L., Sankaran, K., Yager, J. Y., & Martin, R. H. (2012). Serum cytokines in a clinical trial of hypothermia for neonatal hypoxic-ischemic encephalopathy. *Journal of cerebral blood flow and metabolism : official journal of the International Society of Cerebral Blood Flow and Metabolism*, *32*(10), 1888–1896. https://doi.org/10.1038/jcbfm.2012.83

Jensen F. E. (2006). Developmental factors regulating susceptibility to perinatal brain injury and seizures. *Current opinion in pediatrics*, *18*(6), 628–633. https://doi.org/10.1097/MOP.0b013e328010c536

Jensen, E. C., Bennet, L., Hunter, C. J., Power, G. C., & Gunn, A. J. (2006). Post-hypoxic hypoperfusion is associated with suppression of cerebral metabolism and increased tissue oxygenation in near-term fetal sheep. *The Journal of physiology*, *572*(Pt 1), 131–139.

Jiao, M., Li, X., Chen, L., Wang, X., Yuan, B., Liu, T., Dong, Q., Mei, H., & Yin, H. (2020). Neuroprotective effect of astrocyte-derived IL-33 in neonatal hypoxic-ischemic brain injury. *Journal of neuroinflammation*, *17*(1), 251. https://doi.org/10.1186/s12974-020-01932-z

Jin, Y., Silverman, A. J., & Vannucci, S. J. (2009). Mast cells are early responders after hypoxia-ischemia in immature rat brain. *Stroke*, *40*(9), 3107–3112. https://doi.org/10.1161/STROKEAHA.109.549691

Johnston M. V. (2001). Excitotoxicity in neonatal hypoxia. *Mental retardation and developmental disabilities research reviews*, *7*(4), 229–234. https://doi.org/10.1002/mrdd.1032

Kaukola, T., Satyaraj, E., Patel, D. D., Tchernev, V. T., Grimwade, B. G., Kingsmore, S. F., Koskela, P., Tammela, O., Vainionpää, L., Pihko, H., Äärimaa, T., & Hallman, M. (2004). Cerebral palsy is characterized by protein mediators in cord serum. *Annals of neurology*, *55*(2), 186–194. https://doi.org/10.1002/ana.10809

Kaur, C., Rathnasamy, G., & Ling, E. A. (2013). Roles of activated microglia in hypoxia induced neuroinflammation in the developing brain and the retina. *Journal of neuroimmune*

pharmacology : the official journal of the Society on NeuroImmune Pharmacology, *8*(1), 66–78. https://doi.org/10.1007/s11481-012-9347-2

Keller, M., Simbruner, G., Górna, A., Urbanek, M., Tinhofer, I., Griesmaier, E., . . . Gressens, P. (2006). Systemic Application of Granulocyte-Colony Stimulating Factor and Stem Cell Factor Exacerbates Excitotoxic Brain Injury in Newborn Mice. *Pediatric Research, 59*(4), 549-553. doi:10.1203/01.pdr.0000205152.38692.81

Lau, L. T., & Yu, A. C. (2001). Astrocytes produce and release interleukin-1, interleukin-6, tumor necrosis factor alpha and interferon-gamma following traumatic and metabolic injury. *Journal of neurotrauma*, *18*(3), 351–359. https://doi.org/10.1089/08977150151071035

Lazovic, J., Basu, A., Lin, H. W., Rothstein, R. P., Krady, J. K., Smith, M. B., & Levison, S. W. (2005). Neuroinflammation and both cytotoxic and vasogenic edema are reduced in interleukin-1 type 1 receptor-deficient mice conferring neuroprotection. *Stroke*, *36*(10), 2226–2231. https://doi.org/10.1161/01.STR.0000182255.08162.6a

Lee, A. C. C., Kozuki, N., Blencowe, H., Vos, T., Bahalim, A., Darmstadt, G. L., . . . Lawn, J. E. (2013). Intrapartum-related neonatal encephalopathy incidence and impairment at regional and global levels for 2010 with trends from 1990. *Pediatric Research, 74*(1), 50-72. doi:10.1038/pr.2013.206

Leonardo, C. C., & Pennypacker, K. R. (2009). Neuroinflammation and MMPs: potential therapeutic targets in neonatal hypoxic-ischemic injury. *Journal of neuroinflammation*, *6*, 13. https://doi.org/10.1186/1742-2094-6-13

Leviton, A., & Gressens, P. (2007). Neuronal damage accompanies perinatal white-matter damage. *Trends in Neurosciences, 30*(9), 473-478. doi:https://doi.org/10.1016/j.tins.2007.05.009

Li, L., Jiang, Q., Zhang, L., Ding, G., Gang Zhang, Z., Li, Q., . . . Chopp, M. (2007). Angiogenesis and improved cerebral blood flow in the ischemic boundary area detected by MRI after administration of sildenafil to rats with embolic stroke. *Brain Research, 1132*, 185-192. doi:https://doi.org/10.1016/j.brainres.2006.10.098

Li, S. J., Liu, W., Wang, J. L., Zhang, Y., Zhao, D. J., Wang, T. J., & Li, Y. Y. (2014). The role of TNF-α, IL-6, IL-10, and GDNF in neuronal apoptosis in neonatal rat with hypoxic-ischemic encephalopathy. *European review for medical and pharmacological sciences*, *18*(6), 905–909.

Lieschke, S., Zechmeister, B., Haupt, M., Zheng, X., Jin, F., Hein, K., Weber, M. S., Hermann, D. M., Bähr, M., Kilic, E., & Doeppner, T. R. (2019). CCL11 Differentially Affects Post-Stroke Brain Injury and Neuroregeneration in Mice Depending on Age. *Cells*, *9*(1), 66. https://doi.org/10.3390/cells9010066

Liu, F., & McCullough, L. D. (2013). Inflammatory responses in hypoxic ischemic encephalopathy. *Acta Pharmacologica Sinica, 34*(9), 1121-1130. doi:10.1038/aps.2013.89

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. *The Journal of Pediatrics, 194*, 67-75.e61. doi:https://doi.org/10.1016/j.jpeds.2017.10.060

McGowan, M. M., O'Kane, A. C., Vezina, G., Chang, T., Bendush, N., Glass, P., Gai, J., Bost, J., Everett, A. D., & Massaro, A. N. (2021). Serial plasma biomarkers of brain injury in infants with neonatal encephalopathy treated with therapeutic hypothermia. *Pediatric research*, *90*(6), 1228–1234. https://doi.org/10.1038/s41390-021-01405-w

McRae, A., Gilland, E., Bona, E., & Hagberg, H. (1995). Microglia activation after neonatal hypoxic-ischemia. *Brain research. Developmental brain research*, *84*(2), 245–252. https://doi.org/10.1016/0165-3806(94)00177-2

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

Murray, D. M. (2019). Chapter 13 - Biomarkers in neonatal hypoxic–ischemic encephalopathy—Review of the literature to date and future directions for research. In L. S. de Vries & H. C. Glass (Eds.), *Handbook of Clinical Neurology* (Vol. 162, pp. 281-293): Elsevier.

Nair, J., & Kumar, V. (2018). Current and Emerging Therapies in the Management of Hypoxic Ischemic Encephalopathy in Neonates. *Children (Basel, Switzerland)*, *5*(7), 99. https://doi.org/10.3390/children5070099

Neubauer, V., Wegleiter, K., Posod, A., Urbanek, M., Wechselberger, K., Kiechl-Kohlendorfer, U., Keller, M., & Griesmaier, E. (2016). Delayed application of the haematopoietic growth factors G-CSF/SCF and FL reduces neonatal excitotoxic brain injury. *Brain research*, *1634*, 94–103. https://doi.org/10.1016/j.brainres.2015.12.058

Northington, F. J., Chavez-Valdez, R., & Martin, L. J. (2011). Neuronal cell death in neonatal hypoxia-ischemia. *Annals of neurology*, *69*(5), 743–758. https://doi.org/10.1002/ana.22419

Nunes, A. K. S., Rapôso, C., Rocha, S. W. S., Barbosa, K. P. d. S., de Almeida Luna, R. L., da Cruz-Höfling, M. A., & Peixoto, C. A. (2015). Involvement of AMPK, IKβα-NFκB and eNOS in the sildenafil anti-inflammatory mechanism in a demyelination model. *Brain Research, 1627*, 119-133. doi:https://doi.org/10.1016/j.brainres.2015.09.008

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

Osredkar, D., Thoresen, M., Maes, E., Flatebø, T., Elstad, M., & Sabir, H. (2014). Hypothermia is not neuroprotective after infection-sensitized neonatal hypoxic-ischemic brain injury. *Resuscitation*, *85*(4), 567–572. https://doi.org/10.1016/j.resuscitation.2013.12.006

Palmer, C., Roberts, R. L., & Young, P. I. (2004). Timing of Neutrophil Depletion Influences Long-Term Neuroprotection in Neonatal Rat Hypoxic-Ischemic Brain Injury. *Pediatric Research, 55*(4), 549-556. doi:10.1203/01.PDR.0000113546.03897.FC

Parajuli, B., Horiuchi, H., Mizuno, T., Takeuchi, H., & Suzumura, A. (2015). CCL11 enhances excitotoxic neuronal death by producing reactive oxygen species in microglia. *Glia*, *63*(12), 2274–2284. https://doi.org/10.1002/glia.22892

Pauliah, S. S., Shankaran, S., Wade, A., Cady, E. B., & Thayyil, S. (2013). Therapeutic hypothermia for neonatal encephalopathy in low- and middle-income countries: a systematic review and meta-analysis. *PloS one*, *8*(3), e58834. https://doi.org/10.1371/journal.pone.0058834

Peixoto, C. A., Nunes, A. K., & Garcia-Osta, A. (2015). Phosphodiesterase-5 Inhibitors: Action on the Signaling Pathways of Neuroinflammation, Neurodegeneration, and

Cognition. *Mediators of inflammation*, *2015*, 940207. https://doi.org/10.1155/2015/940207

Perlman, J. M. (1997). Intrapartum Hypoxic-Ischemic Cerebral Injury and Subsequent Cerebral Palsy: Medicolegal Issues. *Pediatrics, 99*(6), 851-859. doi:10.1542/peds.99.6.851

Polderman K. H. (2008). Induced hypothermia and fever control for prevention and treatment of neurological injuries. *Lancet (London, England)*, *371*(9628), 1955–1969. https://doi.org/10.1016/S0140-6736(08)60837-5

Ramaswamy, V., Horton, J., Vandermeer, B., Buscemi, N., Miller, S., & Yager, J. (2009). Systematic Review of Biomarkers of Brain Injury in Term Neonatal Encephalopathy. *Pediatric Neurology, 40*(3), 215-226. doi:https://doi.org/10.1016/j.pediatrneurol.2008.09.026

Rathnasamy, G., Ling, E. A., & Kaur, C. (2011). Iron and iron regulatory proteins in amoeboid microglial cells are linked to oligodendrocyte death in hypoxic neonatal rat periventricular white matter through production of proinflammatory cytokines and reactive oxygen/nitrogen species. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *31*(49), 17982–17995. https://doi.org/10.1523/JNEUROSCI.2250-11.2011

Roohey, T., Raju, T. N. K., & Moustogiannis, A. N. (1997). Animal models for the study of perinatal hypoxic-ischemic encephalopathy: a critical analysis. *Early Human Development, 47*(2), 115-146. doi:https://doi.org/10.1016/S0378-3782(96)01773-2

Roy-O'Reilly, M., Ritzel, R. M., Conway, S. E., Staff, I., Fortunato, G., & McCullough, L. D. (2017). CCL11 (Eotaxin-1) Levels Predict Long-Term Functional Outcomes in Patients

Following Ischemic Stroke. *Translational stroke research*, *8*(6), 578–584. https://doi.org/10.1007/s12975-017-0545-3

Russell, T. D., Yan, Q., Fan, G., Khalifah, A. P., Bishop, D. K., Brody, S. L., & Walter, M. J. (2003). IL-12 p40 Homodimer-Dependent Macrophage Chemotaxis and Respiratory Viral Inflammation Are Mediated through IL-12 Receptor β1. *The Journal of Immunology, 171*(12), 6866. doi:10.4049/jimmunol.171.12.6866

Russo Matthew, V., & McGavern Dorian, B. (2016). Inflammatory neuroprotection following traumatic brain injury. *Science, 353*(6301), 783-785. doi:10.1126/science.aaf6260

Sarnat, H. B., & Sarnat, M. S. (1976). Neonatal Encephalopathy Following Fetal Distress: A Clinical and Electroencephalographic Study. *Archives of Neurology, 33*(10), 696-705. doi:10.1001/archneur.1976.00500100030012

Sävman, K., Blennow, M., Gustafson, K., Tarkowski, E., & Hagberg, H. (1998). Cytokine response in cerebrospinal fluid after birth asphyxia. *Pediatric research*, *43*(6), 746–751. https://doi.org/10.1203/00006450-199806000-00006

Shalak, L., & Perlman, J. M. (2004). Hypoxic–ischemic brain injury in the term infant-current concepts. *Early Human Development, 80*(2), 125-141. doi:https://doi.org/10.1016/j.earlhumdev.2004.06.003

Shankaran S. (2012). Therapeutic hypothermia for neonatal encephalopathy. *Current treatment options in neurology*, *14*(6), 608–619. https://doi.org/10.1007/s11940-012-0200-y

Shankaran, S., Laptook, A. R., Ehrenkranz, R. A., Tyson, J. E., McDonald, S. A., Donovan, E. F., Fanaroff, A. A., Poole, W. K., Wright, L. L., Higgins, R. D., Finer, N. N., Carlo, W. A., Duara, S., Oh, W., Cotten, C. M., Stevenson, D. K., Stoll, B. J., Lemons, J. A., Guillet, R., Jobe, A. H., … National Institute of Child Health and Human Development Neonatal

Research Network (2005). Whole-body hypothermia for neonates with hypoxic-ischemic encephalopathy. *The New England journal of medicine*, *353*(15), 1574–1584. https://doi.org/10.1056/NEJMcps050929

Shin, C. Y., Lee, W. J., Choi, J. W., Choi, M. S., Ryu, J. R., Oh, S. J., Cheong, J. H., Choi, E. Y., & Ko, K. H. (2007). Down-regulation of matrix metalloproteinase-9 expression by nitric oxide in lipopolysaccharide-stimulated rat primary astrocytes. *Nitric oxide : biology and chemistry*, *16*(4), 425–432. https://doi.org/10.1016/j.niox.2007.03.004

Silver, B., McCarthy, S., Lu, M., Mitsias, P., Russman, A. N., Katramados, A., . . . Chopp, M. (2009). Sildenafil Treatment of Subacute Ischemic Stroke: A Safety Study at 25-mg Daily for 2 Weeks. *Journal of Stroke and Cerebrovascular Diseases, 18*(5), 381-383. doi:https://doi.org/10.1016/j.jstrokecerebrovasdis.2009.01.007

Simonca, L., & Tulloh, R. (2017). Sildenafil in Infants and Children. *Children (Basel, Switzerland)*, *4*(7), 60. https://doi.org/10.3390/children4070060

Sivakumar, V., Ling, E. A., Lu, J., & Kaur, C. (2010). Role of glutamate and its receptors and insulin-like growth factors in hypoxia induced periventricular white matter injury. *Glia*, *58*(5), 507–523. https://doi.org/10.1002/glia.20940

Sughrue, M. E., Mehra, A., Connolly, E. S., & D'Ambrosio, A. L. (2004). Anti-adhesion molecule strategies as potential neuroprotective agents in cerebral ischemia: A critical review of the literature. *Inflammation Research, 53*(10), 497-508. doi:10.1007/s00011-004-1282-0

Szaflarski, J., Burtrum, D., & Silverstein, F. S. (1995). Cerebral hypoxia-ischemia stimulates cytokine gene expression in perinatal rats. *Stroke*, *26*(6), 1093–1100. https://doi.org/10.1161/01.str.26.6.1093

Vasiljevic, B., Maglajlic-Djukic, S., Gojnic, M., Stankovic, S., Ignjatovic, S., & Lutovac, D. (2011). New insights into the pathogenesis of perinatal hypoxic-ischemic brain injury. *Pediatrics international : official journal of the Japan Pediatric Society*, *53*(4), 454–462. https://doi.org/10.1111/j.1442-200X.2010.03290.x

Volpe J. J. (2012). Neonatal encephalopathy: an inadequate term for hypoxic-ischemic encephalopathy. *Annals of neurology*, *72*(2), 156–166. https://doi.org/10.1002/ana.23647

Volpe, J. J. (2008). Neurology of the newborn E-book.

Wakabayashi, K., Isozaki, T., Tsubokura, Y., Fukuse, S., & Kasama, T. (2021). Eotaxin-1/CCL11 is involved in cell migration in rheumatoid arthritis. *Scientific Reports, 11*(1), 7937. doi:10.1038/s41598-021-87199-7

Wakita, H., Tomimoto, H., Akiguchi, I., Lin, J.-X., Ihara, M., Ohtani, R., & Shibata, M. (2003). Ibudilast, a phosphodiesterase inhibitor, protects against white matter damage under chronic cerebral hypoperfusion in the rat. *Brain Research, 992*(1), 53-59. doi:https://doi.org/10.1016/j.brainres.2003.08.028

Walsh, B. H., Boylan, G. B., Livingstone, V., Kenny, L. C., Dempsey, E. M., & Murray, D. M. (2013). Cord Blood Proteins and Multichannel-Electroencephalography in Hypoxic-Ischemic Encephalopathy*. *Pediatric Critical Care Medicine, 14*(6).

Walter, M. J., Kajiwara, N., Karanja, P., Castro, M., & Holtzman, M. J. (2001). Interleukin 12 P40 Production by Barrier Epithelial Cells during Airway Inflammation. *Journal of Experimental Medicine, 193*(3), 339-352. doi:10.1084/jem.193.3.339

Wang, F., Baba, N., Shen, Y., Yamashita, T., Tsuru, E., Tsuda, M., . . . Sagara, Y. (2017). CCL11 promotes migration and proliferation of mouse neural progenitor cells. *Stem Cell Research & Therapy, 8*(1), 26. doi:10.1186/s13287-017-0474-9

Weber, N. C., Blumenthal, S. B., Hartung, T., Vollmar, A. M., & Kiemer, A. K. (2003). ANP inhibits TNF-alpha-induced endothelial MCP-1 expression--involvement of p38 MAPK and MKP-1. *Journal of leukocyte biology*, *74*(5), 932–941. https://doi.org/10.1189/jlb.0603254

Wintermark, P. (2011). Current Controversies in Newer Therapies to Treat Birth Asphyxia. *International Journal of Pediatrics, 2011*, 848413. doi:10.1155/2011/848413

Wintermark, P., Boyd, T., Gregas, M. C., Labrecque, M., & Hansen, A. (2010). Placental pathology in asphyxiated newborns meeting the criteria for therapeutic hypothermia. *American journal of obstetrics and gynecology*, *203*(6), 579.e1–579.e5799. https://doi.org/10.1016/j.ajog.2010.08.024

Wintermark, P., Hansen, A., Soul, J., Labrecque, M., Robertson, R. L., & Warfield, S. K. (2011). Early versus late MRI in asphyxiated newborns treated with hypothermia. *Archives of disease in childhood. Fetal and neonatal edition*, *96*(1), F36–F44. https://doi.org/10.1136/adc.2010.184291

Wisnowski, J. L., Wintermark, P., Bonifacio, S. L., Smyser, C. D., Barkovich, A. J., Edwards, A. D., de Vries, L. S., Inder, T. E., Chau, V., & Newborn Brain Society Guidelines and Publications Committee (2021). Neuroimaging in the term newborn with neonatal encephalopathy. *Seminars in fetal & neonatal medicine*, *26*(5), 101304. https://doi.org/10.1016/j.siny.2021.101304

Wood, T. R., Vu, P. T., Comstock, B. A., Law, J. B., Mayock, D. E., Heagerty, P. J., Burbacher, T., Bammler, T. K., & Juul, S. E. (2021). Cytokine and chemokine responses to injury and treatment in a nonhuman primate model of hypoxic-ischemic encephalopathy treated with hypothermia and erythropoietin. *Journal of cerebral blood flow and metabolism :*

official journal of the International Society of Cerebral Blood Flow and Metabolism, *41*(8), 2054–2066. https://doi.org/10.1177/0271678X21991439

Wyatt, J. S., Edwards, A. D., Azzopardi, D., & Reynolds, E. O. (1989). Magnetic resonance and near infrared spectroscopy for investigation of perinatal hypoxic-ischaemic brain injury. *Archives of Disease in Childhood, 64*(7 Spec No), 953. doi:10.1136/adc.64.7_Spec_No.953

Yazdani, A., Howidi, B., Shi, M. Z., Tugarinov, N., Khoja, Z., & Wintermark, P. (2021). Sildenafil improves hippocampal brain injuries and restores neuronal development after neonatal hypoxia–ischemia in male rat pups. *Scientific Reports, 11*(1), 22046. doi:10.1038/s41598-021-01097-6

Yazdani, A., Khoja, Z., Johnstone, A., Dale, L., Rampakakis, E., & Wintermark, P. (2016). Sildenafil Improves Brain Injury Recovery following Term Neonatal Hypoxia-Ischemia in Male Rat Pups. *Developmental neuroscience*, *38*(4), 251–263. https://doi.org/10.1159/000448327

Yong, V. W., Power, C., Forsyth, P., & Edwards, D. R. (2001). Metalloproteinases in biology and pathology of the nervous system. *Nature reviews. Neuroscience*, *2*(7), 502–511. https://doi.org/10.1038/35081571

Zhang, J., Guo, J., Zhao, X., Chen, Z., Wang, G., Liu, A., . . . Wang, C. (2013). Phosphodiesterase-5 inhibitor sildenafil prevents neuroinflammation, lowers beta-amyloid levels and improves cognitive performance in APP/PS1 transgenic mice. *Behavioural Brain Research, 250*, 230-237. doi:https://doi.org/10.1016/j.bbr.2013.05.017

Zhang, R., Wang, Y., Zhang, L., Zhang, Z., Tsang, W., Lu, M., . . . Chopp, M. (2002). Sildenafil (Viagra) Induces Neurogenesis and Promotes Functional Recovery After Stroke in Rats. *Stroke, 33*(11), 2675-2680. doi:10.1161/01.STR.0000034399.95249.59

Zhao, S., Zhang, L., Lian, G., Wang, X., Zhang, H., Yao, X., . . . Wu, C. (2011). Sildenafil attenuates LPS-induced pro-inflammatory responses through down-regulation of intracellular ROS-related MAPK/NF-κB signaling pathways in N9 microglia. *International Immunopharmacology, 11*(4), 468-474. doi:https://doi.org/10.1016/j.intimp.2010.12.017

Ziliotto, N., Bernardi, F., Jakimovski, D., Baroni, M., Bergsland, N., Ramasamy, D. P., . . . Ramanathan, M. (2018). Increased CCL18 plasma levels are associated with neurodegenerative MRI outcomes in multiple sclerosis patients. *Multiple Sclerosis and Related Disorders, 25*, 37-42. doi:https://doi.org/10.1016/j.msard.2018.07.009