# Magnesium Sulphate Neuroprotection in Neonatal Encephalopathy

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## **Declaration**

I, Ingran Lingam confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

## **<u>Acknowledgements</u>**

## **Contributions**

Experiments were performed by Adnan Advic-Belltheus, Chris Meehan, Kathryn Martinello, Sara Ragab and Ingran Lingam with support from Nicola Robertson. Technical support for Broadband NIRS was provided by Pardis Kaynezhad, Cornelius Bauer and Ilias Tachtsidis.

Amplitude-integrated EEG data analysis was performed independently by Kathryn Martinello and Ingran Lingam. MRS biomarker quantification was analysed by a team of physicists, including Alan Bainbridge, Magdelena Sokolska and David Price. Immunohistochemistry staining was led by Mariya Hristova and cell counts were undertaken by Chris Meehan and Ingran Lingam. Statistical analysis of aEEG, MRS, immunohistochemistry and gene expression was performed with the support of a medical statistician, Debbie Kraus.

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# Abstract

The efficacy of therapeutic hypothermia (HT) in neonatal encephalopathy (NE) is incomplete; mortality and morbidity remain high at almost 50% despite treatment. This is likely due to both limitations in hypothermic neuroprotection and heterogeneity in aetiology. Improving hypothermia therefore requires both adjunctive neuroprotective agents as well as improved diagnostic tools to differentiate injury subtypes.

In Part (I), we established the safety and efficacy of $MgSO_4$ combined with HT (Mg+HT; n=8) compared to HT (HT; n=7) in piglets after hypoxia ischaemia (HI). $MgSO_4$ (180mg/kg bolus; 8mg/kg/h infusion) was administered 1h post-HI and HT for 12h. In Part (II), we explored whether $MgSO_4$ attenuates cytokine gene expression in the same animals.

Inflammation exacerbates brain injury, therefore anti-inflammatory agents such as $MgSO_4$ may be particularly efficacious in patients with prior inflammation-sensitisation. There are however no biomarkers available to identify this patient subgroup. In Part (III), we utilised advanced biological techniques to distinguish hypoxia (Hypoxia; n=6), inflammation (LPS; n=5) and inflammation-sensitised hypoxia (LPS+Hypoxia; n=5) in a piglet model of inflammation-sensitised NE. LPS was commenced 4h prior to hypoxia (2μg/kg bolus; 1μg/kg infusion).

We demonstrated that $MgSO_4$ bolus and infusion provided a stable, raised serum magnesium without significant hypotension in hypothermic piglets post-HI. $MgSO_4$ reduced overall cell death, however did not demonstrate a significant reduction in the primary outcome of MRS Lac/NAA. This suggests that the observed improvement was incremental and most likely insufficient to provide long-term benefit.

$MgSO_4$ did not alter microglial activation or astrogliosis, suggesting combination therapy did not attenuate inflammation post-HI. Consistent with this finding, magnesium exposure did not alter serum mRNA levels of inflammatory cytokines, chemokines or inducible nitric oxide synthetase. Whilst there is insufficient evidence to translate $MgSO_4$ to clinical trials, magnesium remains an agent of interest which may yet find its place as part of a cocktail of neuroprotective medications that work incrementally and synergistically.

## Impact Statement

Neonatal encephalopathy (NE) represents a significant global burden and is the second leading cause of mortality in infants under 28 days. Despite the introduction of therapeutic hypothermia, almost half of infants still develop adverse neurological sequelae. Attempts to further optimise hypothermia by cooling to deeper temperatures (32°C) for longer durations (120 hours) have failed to improve upon standard therapy.

The primary aim of this thesis is to improve neurological injury following perinatal asphyxia through assessing the efficacy of adjunctive pharmacological agents given alongside hypothermia; as well as exploring methods to further define the underlying pathological processes occurring in infants with NE. Magnesium is an attractive neuroprotective agent due to its widespread availability, low cost and safety profile. This study is the first term-equivalent large animal study to explore the safety and efficacy of a magnesium sulphate bolus and infusion in combination with hypothermia.

Inflammation exposure is well known to exacerbate brain injury and potentially reduces the effectiveness of hypothermia. With the shift towards precision medicine with treatments tailored to the underlying disease process, there is a great need to identify those infants with inflammation-sensitised hypoxic injury. Currently, there are no studies examining biomarkers that differentiate inflammation from hypoxic brain injury.

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This thesis uses a well-established piglet model of NE to deliver a reproducible, standardised HI injury to assess the efficacy of magnesium sulphate in combination with hypothermia. The translatable nature of this experimental model and use of clinically relevant outcome measurements will facilitate transfer of these findings to clinical practice.

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## Abbreviations

| | |
|---|---|
| $^1$H MRS | Proton Magnetic Resonance Spectroscopy |
| $^{31}$P MRS | Phosphorus Magnetic Resonance Spectroscopy |
| aEEG | Amplitude-integrated EEG |
| AMPA | α-amino-3-hydroxy-5-methylisoazole-4- propionic acid |
| ARRIVE | Animal Research: Reporting of *In Vivo* Experiments |
| AUC | Area Under Curve |
| AWERB | Animal Welfare Ethical Review Body |
| BBB | Blood Brain Barrier |
| BDNF | Brain Derived Neurotrophic Factor |
| BE | Base Excess |
| BGT | Basal Ganglia / Thalamus |
| $Ca^{2+}$ | Calcium Ion |
| CC3 | Cleaved Caspase 3 |
| cDNA | Complementary DNA |
| CI | Confidence Interval |
| CNS | Central Nervous System |
| CSF | Cerebrospinal Fluid |
| DALY | Disability Life Adjusted Years |
| DNA | Deoxyribonucleic Acid |
| EDTA | Ethylenediaminetetraacetic acid |
| EEG | Electroencephalogram |
| ENO2 | Enolase 2 |
| $H_2O_2$ | Hydrogen Peroxide |
| HbD | Difference in Haemoglobin |
| $HbO_2$ | Oxyhaemoglobin |
| HbT | Total Haemoglobin |
| $HCO_3$ | Bicarbonate |
| HHb | Deoxyhaemoglobin |
| HI | Hypoxia Ischaemia |
| HIE | Hypoxic Ischaemic Encephalopathy |
| HR | Heart Rate |
| HSA | *Homo Sapiens* species |
| HT | Hypothermia |
| IBA1 | Ionised calcium binding adaptor molecule 1 |
| ICAM-1 | Intercellular Adhesion Molecule 1 |
| IL | Interleukin |
| INF$\gamma$ | Interferon gamma |
| IQ | Intelligence Quotient |
| IU | International Unit |
| KA | Kainic Acid |
| Lac | Lactate |
| LPS | Lipopolysaccharide |
| MABP | Mean Arterial Blood Pressure |
| MAP2 | Microtubule associated protein 2 |
| MAPT (Tau) | Microtubule-associated Protein Tau (gene coding for Tau protein) |
| MBP | Myelin Basic Protein |
| MCAO | Middle cerebral artery occlusion |
| MCP1 | Monocyte Chemoattractant protein 1 |
| $MgSO_4$ | Magnesium Sulphate |
| miRNA / miR | MicroRNA |
| MMP9 | Matrix Metallopeptidase 9 |
| MRI / MRS | Magnetic Resonance Imaging / Magnetic Resonance Spectroscopy |
| mRNA | Messenger RNA |
| NAA | N-acetyl aspartate |
| NE | Neonatal Encephalopathy |
| NF-κB | Nuclear Factor Kappa B |
| NIRS | Near Infrared Spectroscopy |

| | |
|---|---|
| NMDA | N-methyl-D-aspartate |
| NNT | Number Needed to Treat |
| NO | Nitric Oxide |
| NR2 | NMDA receptor subunit 2 |
| NSE | Neuron Specific Enolase (encoded in ENO2 gene) |
| NTP | Nucleotide Triphosphate |
| $O^{2-}$ | Superoxide radical |
| OLIG2 | Oligodendrocyte Transcription Factor 2 |
| OR | Odds Ratio |
| oxCCO | oxidisation of Cytochrome C Oxidase |
| P7 | Postnatal Day 7 |
| PBS | Phosphate-buffered saline |
| pCO2 | Partial Pressure of Carbon Dioxide |
| PCr | Phosphocreatine |
| PFA | Paraformaldehyde |
| $pH_i$ | Intracerebral pH |
| $P_i$ | Inorganic phosphate |
| PPIA | Peptidylprolyl isomerase A |
| RNA | Ribonucleic Acid |
| ROS | Reactive Oxygen Species |
| RR | Relative Risk |
| RT | Reverse Transcriptase |
| S100B | S100 calcium-binding protein B |
| SOD | Superoxide Dismutase |
| SSC | *Sus scrofa* species |
| STAT | Signal transducer and activator of transcription |
| TLR | Toll-like Receptor |
| TNF | Tumour Necrosis Factor |
| TUNEL | Terminal deoxynucleotidyl transferase dUTP nick end labelling |
| UCHL1 | Ubiquitin C-Terminal Hydrolase L1 |
| v/v | Volume / Volume |
| VEGF | Vascular endothelial growth factor |
| VLBW | Very Low Birth Weight |
| WIDEA | Warner Initial Developmental Evaluation of Adaptive and Functional Skills |
| YWHAZ | Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta |

**<u>Chapter 1:</u>**


**Introduction**

## 1.1 Neonatal Encephalopathy

### 1.1.1 Definitions

Neonatal Encephalopathy (NE) was described by Nelson and Leviton as a disorder of term infants in the first few days of life characterised by difficulty in initiating and maintaining respiration, depressed tone and reflexes, reduced consciousness and often seizures [1]. NE encompasses a wide spectrum of aetiologies, including hypoxia ischaemia (HI), intracranial haemorrhage, severe hyperbilirubinaemia, metabolic disturbances and infection [2].

Hypoxic Ischaemic Encephalopathy (HIE) represents a subset of infants with NE, where cerebral injury has been caused by a combination of oxygen deprivation (hypoxia) and interrupted cerebral blood supply (ischaemia). HIE is predominately caused by adverse perinatal events which impair respiratory gas exchange (perinatal asphyxia). Trials assessing neuroprotective interventions recruited infants with evidence of HI, defined by acidotic blood gas analysis or the persistent need for resuscitation in combination with signs and symptoms of neurological compromise.

### 1.1.2 Epidemiology

Population and hospital-based studies estimate the incidence of NE and HIE as between 1 – 8 infants per 1000 live births in high income countries [3], however has also been reported as high as 26.2 per 1000 live births in resource-limited settings [4].  NE represents a significant global health burden

with an estimated 643,765 deaths worldwide and the second leading cause of mortality in infants under 28 days [5,6].  This is very likely an underestimate given the additional 1.3 million stillbirths occurring during labour unaccounted for in the global burden estimation [7].  Despite an overall reduction in global rates of NE in the last 2 decades, progress has been disappointing with a failure to achieve the Millennium Development Goal and NE now representing the 3rd highest Disability Adjusted Life  Years (DALYs) in the under 5s at 56 million [8].

*1.1.3 Classification*

Different classification systems have been developed using clinical criteria to stratify the severity of NE and identify infants at highest risk of adverse outcomes (see Table 1).

NE was first described by Amiel-Tilson in 1969 [9] and later classified into three stages of severity by Sarnat and Sarnat [10]. Stage 1 (mild) was characterised by hyperalertness, sympathetic symptoms, uninhibited primitive reflexes with a normal electroencephalogram (EEG); infants with stage 2 (moderate) were hypotonic, obtunded with strong distal flexion and multifocal seizures; and stage 3 (severe) infants were comatose, hypotonic with suppressed brainstem and autonomic function. The EEG in infants with severe NE was isoelectric or displayed infrequent periodic discharges. Infants were followed up for 6 - 12 months and those with stage 1 or 2 for less than 5 days appeared normal,

whereas infants with ongoing signs of stage 2 or stage 3 NE developed adverse outcomes [10].

Similarly, Levene categorised NE into 3 stages using a modified description by Fenichel [11] which incorporated time constraints to reflect the progressive nature of symptoms over the first 48 hours. Amiel-Tilson further subdivided each of these categories to improve prognostication. Infants with isolated seizures were placed in a more severe category as stage 2B and infants in stage 3 were subdivided into the presence (3A) or absence (3B) of oculovestibular signs.

| | Sarnat | | Levene | | Amiel-Tilson | | | |
|---|---|---|---|---|---|---|---|---|
| | *Stage 2* | *Stage 3* | *Stage 2* | *Stage 3* | *Stage 2A* | *Stage 2B* | *Stage 3A* | *Stage 3B* |
| Tone | ↓ | ↓↓ | ↓ | ↓↓ | ↓ | ↓ | ↓↓ | ↓↓ |
| Consciousness | Lethargic | Obtunded / Coma | Lethargic | Obtunded / Coma | Lethargic | Lethargic | Obtunded/ Coma | Obtunded/ Coma |
| Reflexes (suck, moro) | Reduced | Absent | Reduced | Absent | Reduced | Reduced | Absent | Absent |
| Depressed / Absent respiration | | | | Absent | Spontaneous respiration | | +/- apnoea | Absent |
| Seizures | May be present | May be present | Yes | Prolonged | None | Isolated seizure | Prolonged/ Repetitive | Prolonged/ Repetitive |

***Table 1. Classification of NE***

A simple, quantitative scoring system was developed by Thompson and colleagues [12] and validated in a cohort of 45 term infants with NE. Peak scores of 15 or greater were predictive of neurological outcome at 12 months of age (Table 2).

| Sign | 0 | 1 | 2 | 3 |
|------|---|---|---|---|
| Tone | Normal | Hyper | Hypo | Flaccid |
| LOC | Normal | Hyperalert, stare | Lethargic | Comatose |
| Fits | Normal | < 3 per day | > 2 per day | |
| Posture | Normal | Fisting, cycling | Strong distal flexion | Decerebrate |
| Moro | Normal | Partial | Absent | |
| Grasp | Normal | Poor | Absent | |
| Suck | Normal | Poor | Absent ± bites | |
| Respiration | Normal | Hyperventilation | Brief apnoea | IPPV |
| Fontanelle | Normal | Full, not tense | Tense | |

*Table 2. Thompson Score: a quantitative measure of NE severity. Scoring out of 22; mild 1-10, moderate 11-14 and severe 15-22.*

*1.1.4 Patterns of injury in neonatal encephalopathy*

Heterogeneity in the magnitude, duration and mechanism of HI gives rise to different patterns of brain injury.

Infants exposed to a sentinel event, such as uterine rupture, placental abruption or cord prolapse are likely to develop basal ganglia and thalamic (BGT) injury [13], similar to those observed in early primate models of acute total asphyxia [14]. Lesions in the BGT are often accompanied by abnormal appearances in the posterior limb of the internal capsule and together, are highly predictive of a poor neurological outcome [15]. Term infants with BGT lesions often also have abnormal signal intensity in specific cortical regions, including the central sulcus, interhemispheric fissure and insula.

The majority of fetal distress however, develops without obvious cause and significant BGT injury often occurs in the absence of a well-documented

sentinel event. In fact, Mallard and colleagues demonstrated significant striatal injury with repeated brief episodes of ischaemia in fetal sheep [16].

White matter and cortical injury in the absence of BGT lesions are uncommon and may represent an atypical mechanism of perinatal injury, complicated by periods of hypotension, infection and hypoglycaemia. This is sometimes referred to as a watershed injury, given the selective vulnerability of cortical areas supplied by peripheral branches of both the anterior middle cerebral artery and poster middle cerebral artery. The neurological manifestations of this type of injury may be mild at birth and though severe motor impairment is uncommon, these infants are at long term risk of cognitive delay, language impairment and parieto-occipital epilepsy [17,18].

Perinatal arterial ischaemic strokes (PAIS) share common hypoxic risk factors with HIE. The clinical history in these infants may be complicated, with preceding reduced fetal movements, abnormal cardiotocograph and complex obstetric management [13,19]. Thus these two clinical entities, though considered separate, may occur in tandem.

Our experimental model initiates brain injury using hypoxia and transient cerebral ischaemia for 20-25 minutes. Though no formal radiological reporting of brain MRI has been performed in our studies, scans performed at 24 and 48 hours typically demonstrate severe cerebral oedema consistent with acute injury. The pattern of injury in piglets following HI is illustrated by sampling histological markers of cell death across several key brain regions, showing

greatest injury in the basal ganglia and cortical white matter [20,21]. Of note, the cerebellum has not been assessed in our model.

*1.1.5 Mechanism of injury in neonatal encephalopathy*

Perinatal asphyxia occurs when there is an inadequate supply of oxygen and glucose to meet the metabolic demands of the brain. This may be a result of several different mechanisms, including compromised uterine blood flow (e.g. placental abruption), umbilical circulation (e.g. prolonged labour, cord around neck) or failure to establish breathing after delivery (meconium aspiration) [22]. Brain injury however continues well beyond this sentinel event despite re-establishing adequate oxygenation and perfusion. This is due to a cascade of cellular mechanisms that propagate neuronal cell death in the minutes, hours and days following asphyxia (see Figure 1).
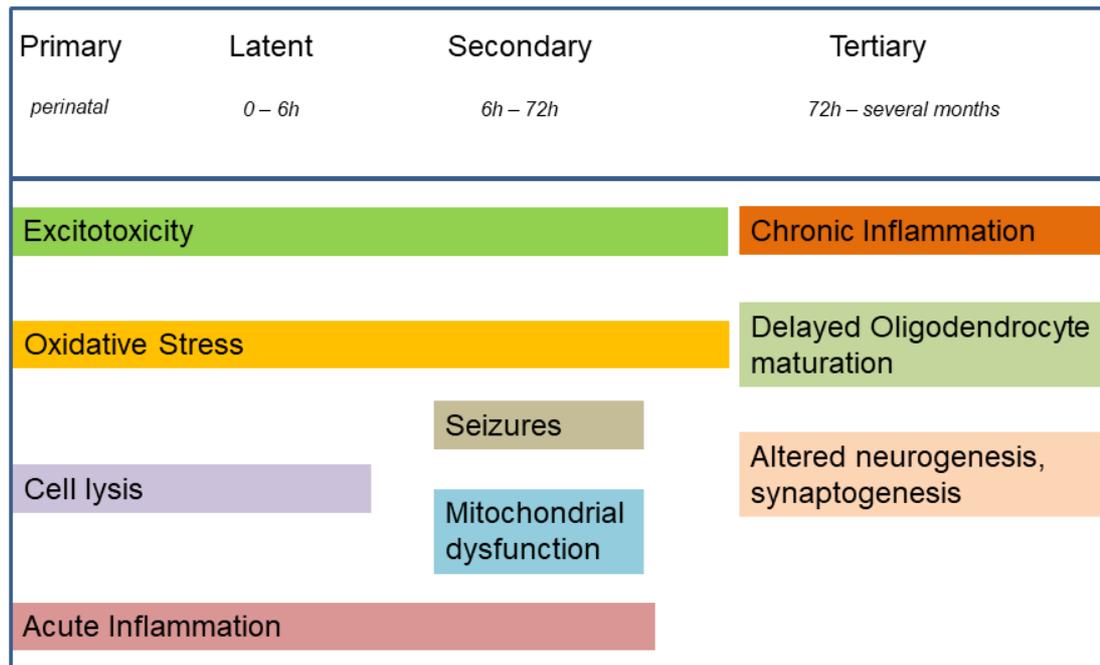
*Figure 1. Phases of neuronal injury in NE*

(a) Primary energy failure

Inadequate oxygen and glucose delivery results in the cessation of oxidative metabolism and depletion of high energy phosphate stores in neuronal cells. This process has been observed *in vivo* in animal models using phosphorus ($^{31}P$) and proton ($^{1}H$) MRS to quantify changes in neurometabolic compounds. Phosphocreatine provides a temporary source of high-energy phosphate to regenerate ATP in an oxygen-restricted environment. During HI, there is an initial fall in phosphocreatine levels (PCr) with a reciprocal rise in inorganic phosphate ($P_i$) resulting in a low PCr:$P_i$ ratio [23]. Nucleotide triphosphate (NTP) levels, the majority of which is ATP, therefore remain stable until phosphocreatine stores have been depleted. The fall in PCr:$P_i$, NTP and intracerebral pH ($pH_i$) therefore represents an interruption in oxidative

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phosphorylation. Cerebral energy manufacture is then reliant on anaerobic glycolysis, resulting in a rise in cerebral lactate.

This initial ("primary") phase of injury results in brain cell death through several different mechanisms;

(i) Cytotoxic oedema

The limited production of ATP is insufficient to maintain cellular homeostasis, including failure of $Na^+/K^+$ ATP-dependent pumps. Depolarisation results in excessive sodium and calcium entry into neurons, which in turn generates an electrical and osmotic gradient, promoting cation and water entry. Subsequent cytotoxic oedema causes cell lysis when severe. This process is reversible and cytotoxic swelling may recover in the first 30 - 60 minutes following reperfusion [24].

(ii) Accumulation of excitatory amino acids and excitotoxicity

Glutamate is the principal excitatory neurotransmitter in the brain and mediates synaptic transmission in multiple neuronal pathways, including learning, memory and somatosensory function [25].

Under normal physiological conditions, glutamate is released following neuronal depolarisation and rapidly returned to the pre-synaptic neuron by high affinity glutamate transporters either directly or via astroglia. Disruption to

the ATP dependent $Na^+/K^+$ pumps and $Na^+$-$Ca^{2+}$ exchangers raises intracellular calcium, causing neuronal depolarisation and excessive glutamate release. This coupled with impaired glutamate reuptake results in the accumulation of excessive extracellular excitatory amino acids [26].

Excessive glutamate release initiates a pathological cascade of injury known as excitotoxicity. This process is mediated by three principal post-synaptic glutamate receptors; N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methylisoazole-4- propionic acid (AMPA) and kainic acid (KA) [25,27].

The NMDA receptor is a heterotetramer, composed of two NR1 subunits and two NR2 subunits akin to a molecule of haemoglobin. The NR1 subunits form the ion channel itself, while the NR2 subunits have a regulatory function and alter the characteristics of the ion channel pore  [27].  A magnesium ion is situated within this calcium ion channel and is displaced on partial depolarisation of the resting membrane. This characteristic enables the receptor to strengthen synaptic connections with repeated activation, a process known as long-term potentiation and an essential mechanism in memory formation. The amino acid glycine is also necessary as a co-agonist for the ion channel to open.  Disruption of membrane potential due to failure of ATP-dependent $Na^+$-$K^+$ pumps displaces this magnesium ion and permits excessive calcium ion entry into the post-synaptic neuron.

AMPA receptors are activated solely by glutamate and primarily control sodium and potassium flux into neuronal cells. Some are permeable to calcium

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depending on their subunit composition [27]. Excessive calcium entry due to AMPA receptor activation also contributes to excitotoxic injury [28].

Raised intracellular calcium acts as a secondary messenger, initiating several injurious enzymes including lipases, proteases, endonucleases and phospholipases. Proteases and endonucleases disrupt cytoskeletal filaments and nuclear proteins. Phospholipid hydrolysis by phospholipases compromises cellular membranes.

(iii) Reactive oxygen species

Reactive oxygen species (ROS), such as superoxide ($O_2$-), hydroxyl radicals (OH) and hydrogen peroxide ($H_2O_2$) are by-products of the electron transport chain in mitochondria and are usually rapidly scavenged by antioxidant enzymes such as superoxide dismutase (SOD). The initial perinatal insult and subsequent reperfusion increases the generation of these ROS, overwhelming the endogenous protective mechanisms. Hydroxyl radicals (OH) cause lipid peroxidation and nucleic acid fragmentation [29]. Nitric oxide free radicals (NO) generated by neuronal nitric oxide synthase (nNOS) during HI react with superoxides in the cytoplasm and mitochondria, producing toxic peroxynitrites and other reactive nitrogen species which damage the cell membrane, organelles and nucleic acids.

(iv) Inflammation

The role of inflammation in the pathogenesis of neuronal injury in NE is discussed in detail in Section 1.2. In brief, neuroinflammation is driven by activated microglia, astrocytes and pro-inflammatory cytokines including interleukin (IL) 1β, tumour necrosis factor (TNF) α and chemotactic factors. Inflammatory cytokines cause injury directly through the production of inducible NOS, cyclooxygenase and ROS; and indirectly by stimulating excitatory amino acid release from glial cells.

The immune response to cerebral injury is complex and not solely pathological. In fact, cortical infarction and behavioural deficits following HI were exacerbated in TNF-knockout mice compared to wild type [30].

(b) Latent Phase

Restoration of oxygenation and perfusion following resuscitation allows oxidative phosphorylation to resume. This recovery in cerebral energetics however is transient and represents the "latent" phase for up to 6 hours post-insult [23]. The cascade of cellular injury during this phase eventually reaches a critical threshold that culminates in the initiation of secondary energy failure.

(c) Secondary energy failure

In moderate and severe cerebral injuries, a progressive "secondary" deterioration occurs over the subsequent 6 to 48 hours post-injury. This is associated with: a fall in NTP and PCr/Pi; rise in intracerebral lactate [23]; reduction in oxidisation of cytochrome c oxidase (indicating a failure of mitochondrial activity); and seizures [31]. The precise mechanism that initiates secondary energy failure remains unclear. It is likely that several factors, including the severity of initial injury, genetics and co-existing inflammatory processes play an important role in determining which infants progress to secondary energy failure.

Although extracellular excitatory amino acids are rapidly cleared following reperfusion [24], hypoxia-induced alterations in AMPA subunit composition [32] and hyperexcitability of the NMDA receptor [33] result in ongoing excessive accumulation of intracellular calcium. This excessive accumulation of calcium eventually overwhelms mitochondrial buffering mechanisms, interrupts oxidative phosphorylation and generates ROS. Progressive mitochondrial dysfunction leads to leakage of cytochrome c and other pro-apoptotic proteins into the cytosol which initiates cell death. Further injury is generated by inflammation, including cytokine-mediated injury and activation of microglia, astrocytes and systemic leukocytes.

(d) Tertiary energy failure

Chronic inflammation represents a third phase of injury following perinatal asphyxia and may persist for several months. Increased microglial activation was noted 12 months following traumatic brain injury in non-human primates [34] and microglial expression of pro-inflammatory cytokines continued for 7 months following irradiation-induced injury [35]. Prolonged inflammation interrupts normal neurodevelopmental processes; adversely affecting synaptic pruning, neurogenesis and memory [36]. Acetylation, a mechanism of post-transcriptional gene expression, is reduced following HI [37] and is associated with cognitive decline [36]. MicroRNAs (miRNA), non-coding segments of ribonucleic acids that regulate gene expression, are significantly altered following HI and these changes persist as long as one year afterwards [38]. Altered acetylation and miRNA expression are also thought to delay oligodendrocyte differentiation and maturation, increasing the vulnerability of white matter to hypoxic injury.

*1.1.6 Apoptosis, necrosis and autophagy*

Mechanisms of cell death are often classified into discrete categories - necrosis, apoptosis and autophagy. Classically, necrosis is described as a rapid process whereby failure to maintain cellular homeostasis results in cytotoxic oedema, loss of membrane integrity and cell lysis. Apoptosis however is an energy-dependent form of programmed cell death characterised by cell shrinkage, chromatin condensation and DNA fragmentation.

Apoptosis may be initiated by caspase 9 following translocation of mitochondrial cytochrome c into the cytoplasm (intrinsic pathway) or caspase 8 by cell membrane death receptors such as TNF receptor (extrinsic pathway) [39]. Activation of either caspase 8 or 9 (initiator caspases) leads to cleavage of caspase 3 (effector caspase) which activates pro-apoptotic protein kinases and disables DNA repair mechanisms.

Apoptosis and necrosis however exist as a continuum with hybrid forms of cell death sharing morphological and molecular features. The predominant mode of cell death is influenced by several factors, including injury severity, brain maturity as well as the subtype of excitatory glutamate receptor activated. Higher severity of injury is associated with necrosis, whereas milder insults favour an apoptotic cell death phenotype.

Autophagy refers to the cellular mechanism to recycle intracellular macromolecules and organelles through lysosomal degradation. Though autophagy may be a normal process to maintain homeostasis in cells undergoing physiological stress, it has also been noted to trigger a form of programmed cell death that is distinct from apoptosis in response to hypoxia [40], excitotoxicity [41] and focal cerebral ischaemia [42]. Autophagosome formation and neuronal cell death was significantly increased in pyramidal neurons of mice undergoing HI and deletion of the Atg7 gene (essential for autophagy induction) was neuroprotective [43]. Conversely, rapamycin-induced autophagy decreased necrotic cell death 24 hours following HI in neonatal rats [44]. This highlights the dual nature of many of the biological

processes that underpin injury progression, possessing both pathological and protective roles depending on the region, timing and severity of injury [39].

*1.1.7 Therapeutic hypothermia*

(a) Evidence of efficacy

There is extensive pre-clinical and clinical evidence that moderate hypothermia (33-34°C) for 72 hours reduces cerebral injury following moderate to severe NE. The first large randomised control trial was undertaken in New Zealand; the Coolcap [45] trial assessed the efficacy of selective head cooling with mild systemic hypothermia. Subsequent studies of total body hypothermia were performed by the National Institute of Child Health and Human Development (NICHD) Neonatal research network [46] (United States), the TOBY trial [47] (UK), Neo.nEURO [48] (Europe), ICE [49] (Australia, New Zealand and US) and a randomised control trial in China [50].

Meta-analysis [51] demonstrated that moderate hypothermia significantly reduced the risk of death and major neurodisability at 18 months (typical RR 0.75, 95% CI 0.68 - 0.83). Importantly, therapeutic hypothermia reduced the risk of death without increasing major disability in the survivors. Selective head cooling demonstrated a non-significant reduction in mortality (RR 0.78, 95% CI 0.59 - 1.04) and major neurodisability (RR 0.72, 95% CI 0.5 - 1.05). Its efficacy was greatest in infants with moderate HIE with a trend towards

improvement in severe cases, most likely due to the fewer number of infants in this category.

The NICHD cohort were followed up to school age (6 - 7 years) [52] and the reduction in mortality and composite mortality and/or severe disability (IQ < 55, blindness or GMFCS IV - V) persistent into childhood. The previously observed reduction in death and/or moderate to severe disability however lost statistical significance in the older age group. The efficacy of cooling in the TOBY cohort [53] however appeared to persist into childhood with significantly higher disability-free survival (defined as IQ > 85 and no neurological abnormality) in the cooled group (RR 1.31, 95%CI 1.01-1.71) and a significant reduction in the risk of cerebral palsy and risk of moderate or severe disability.

(b) The therapeutic window

The efficacy of therapeutic hypothermia is time critical. Near term fetal sheep undergoing 30 minutes of cerebral HI demonstrated a dramatic reduction in neuronal and white matter loss following 72 hours of selective head cooling when initiated 90 minutes following the sentinel event [54]. Delaying hypothermia by 5.5 hours, prior to the onset of secondary seizures, provided partial neuroprotection [55,56]. Efficacy however was no longer apparent when treatment commenced 8.5 hours after index hypoxia in near term fetal lambs [57]. This therapeutic window coincides with the 'latent' phase of injury.

Clinical studies confirm that earlier initiation of therapeutic hypothermia (< 3 hours) significantly improves motor outcomes compared to later initiation (3 – 6 hours) [58]. It is reasonable to assume additional therapies given alongside hypothermia designed to attenuate secondary energy failure should also be given as early as possible following perinatal asphyxia.

(c) Mechanisms of action

Despite clear evidence of efficacy, the precise mechanisms of hypothermic neuroprotection remain unclear. Microarray analysis of global brain tissue gene expression of rodents following focal cerebral ischaemia identified 50 genes upregulated and 103 genes downregulated in rodents treated with hypothermia, highlighting the complexity of neuroprotective mechanisms induced by this treatment. Pathway analysis implicated several genes associated with hypothermia, including protein synthesis, calcium homeostasis, inflammation, cell death and apoptosis.

Experimental data suggests hypothermia reduces excitotoxic brain injury. Brief forebrain ischaemia in gerbils resulted in downregulation of the gluR2 subunit of the AMPA receptor, increasing its permeability to calcium ions and therefore increasing cell death. Hypothermia for 48 hours initiated at 1 hour post-insult significantly attenuated this decline in GluR2 mRNA expression and promoted recovery, protecting against the toxic influx of calcium [32]. Excitotoxin levels post-injury however typically normalise within 1 hour of reperfusion and

therefore this mechanism alone does not explain the protective effects of hypothermia.

Hypothermia reduces cerebral energy utilisation by approximately 5% per 1°C drop in temperature [59]. Reduced metabolism and therefore oxygen consumption during reperfusion may suppress the oxygen free radical release and peroxidation of structural cell membrane lipids [60,61].

Mitochondrial integrity is key to maintaining normal cellular energetics and is disrupted during the secondary phase of injury following HI. Release of cytochrome c initiates pro-apoptotic factors that ultimately activate caspase 3 and initiates programmed cell death. Hypothermia during or after HI reduces cytochrome c release in adult rodents [62,63] and suppress mitochondrial permeability in adult minipigs [64]. Measurements of cerebral energetics post-hypoxia in piglet and rodent models demonstrate that hypothermia preserves high-energy phosphate production, indicating rescue of mitochondrial function [65,66].

Prevention of apoptotic pathways is a key feature of hypothermic neuroprotection. Suppression of caspase 3 activation, the final pathway to initiate apoptosis, has been observed following hypothermia in pre-oligodendrocytes [67], P7 rodents [68] and near term fetal sheep [56]. In the newborn piglet, hypothermia reduced apoptosis, though not necrosis [69].

Hypothermia also attenuates the inflammatory response to injury, reducing microglial proliferation, chemotaxis and induction of pro-inflammatory cytokines as well as suppressing nuclear factor kappa B (NFkB), a key transcription factor that activates pro-inflammatory cytokine gene expression [70].

(d) Optimising therapeutic hypothermia

Although there is extensive evidence supporting the neuroprotective efficacy of moderate hypothermia, the overall mortality associated with HIE remains unacceptably high at 25%, with 20% of survivors suffering significant neurological sequelae [51]. There remains therefore, a great need to continue to optimise neurological outcomes in this vulnerable population.

Shankaran and colleagues assessed the efficacy of prolonged (120 hours) and deeper (32$^o$C) hypothermia, however the trial was terminated early due to concerns regarding its safety and futility [71]. Infants cooled for 120 hours had increased frequency of arrhythmias and a prolonged length of stay. Deeper cooling was associated with increased use of inhaled NO, oxygen requirements and ECMO.

## 1.1.8 Other neuroprotective strategies

Given that therapeutic hypothermia has already been implemented at optimum depth and duration, attention has now been directed towards pharmacological adjuncts to incrementally improve neurodevelopmental outcomes.

Erythropoietin (EPO) promotes neurogenesis, decreases apoptosis and has anti-inflammatory properties in pre-clinical models of HI [72]. Two clinical trials of EPO in infants with moderate to severe HIE have demonstrated neurodevelopmental benefit [73,74], however both predated the introduction of therapeutic hypothermia. Currently, two large randomised controlled trials assessing the efficacy of EPO in addition to therapeutic hypothermia have completed enrolment and are awaiting follow up data (Neurepo NCT01732146; Neato NCT01913340).

Dexmedetomidine, a selective α2-adrenoreceptor agonist, is thought to confer neuroprotection through anti-inflammatory mechanisms. Pharmacokinetic data from piglets after HI however highlighted significant cardiovascular compromise associated with drug accumulation [75]. There is limited data available assessing its neuroprotective potential in infants with NE.

The neuroprotective potential of topiramate has been demonstrated in animal models of HI [76]. A feasibility study of topiramate in moderate to severe HIE and cooling (NeoNATI) demonstrated a promising safety profile, however did not show a reduction in mortality or disability [77].

Melatonin in combination with therapeutic hypothermia has shown promise in preclinical models of HI. Our group has recently demonstrated intravenous melatonin improved the neuroprotective benefit of hypothermia when administered 1 hour following HI in newborn piglets [21] (see Appendix A1). A small randomised control trial of oral melatonin with moderate hypothermia in term infants improved survival and developmental outcome at 6 months [78].

## 1.2 Neuro-inflammation in perinatal injury

### 1.2.1 Definition

Inflammation is a complex immunological response to injury and / or infection, involving a network of leukocytes and diverse family of cell signalling molecules, known as cytokines. Cytokines include interleukins (IL), interferons (IFN), tumour necrosis factor (TNF), chemokines and growth factors. Chemokines are chemoattractant; controlling and coordinating leukocyte migration following injury.

### 1.2.2 Inflammation in neonatal encephalopathy

The central nervous system (CNS) undergoes several essential stages of development during fetal and neonatal life. Disruption to this process through direct injury and inflammation has significant long-lasting consequences [79] and has increasingly been implicated in the pathogenesis of NE.

The inflammatory response involves the coordination of several different immune cells. In the uninjured brain, microglia extend and retract their ramifications to inspect the neuronal micro-environment. When activated by a hypoxic ischaemic event, they develop macrophage-like properties including phagocytosis, cytokine production, antigen presentation and release of matrix metalloproteinases (MMPs) which result in the breakdown of the blood brain barrier (BBB) [80]. Entry of peripheral immune cells through the BBB and choroid plexus further exacerbate the inflammatory response [79]. The activation of microglia during the inflammatory cascade is not solely detrimental. Classical activation, or M1 phenotype, is characterised by the production of pro-inflammatory mediators. As disease progresses, microglia also switch to an anti-inflammatory M2 phenotype which promotes resolution of injury, clearance of oxygen free radicals and wound healing. Microglia may switch between M1 and M2 phenotypes depending on inflammatory cell signalling [80]. Microglial activation and infiltration is one of the pathological hallmarks of HI injury; post-mortem examinations from 178 children demonstrated a greater density of microglial infiltrates in the hippocampi of those who died from HIE compared to those who had died from other causes such as trauma or sepsis [81].

Astrocytes, star-shaped glial cells that provide trophic support to neurons, are also activated within minutes of neuronal injury. Similar to microglia, astrocytes can both exacerbate injury through secretion of pro-inflammatory cytokines, including IL1, TNFα and INFγ as well as promote resolution through the

release of anti-oxidative mediators such as glutathione and SOD [82,83]. Intrinsic microglia and astroglia, as well as infiltrating immune cells, produce cytokines and chemokines which together coordinate the initiation, propagation and resolution of inflammation.

Hypoxia ischaemia itself induces an inflammatory reaction in both the brain parenchyma and peripheral immune system. Term infants with HIE had higher levels of serum and CSF pro-inflammatory cytokines (IL1β, TNFα) compared to controls on day 1, 3 and 7 after birth [84]. Umbilical cord levels of IL6 were found to be 376-fold higher in 50 infants with perinatal asphyxia but with no signs of infection compared to controls [85]. Term infants undergoing cardiac surgery demonstrated increased number and activation of peripheral neutrophils and monocytes within minutes of circulatory arrest during cardiopulmonary bypass [86]. Experimental models confirm that the immunological response to HI occurs within hours of injury [87] and can continue for several weeks [36].

Inflammatory cytokines and chemokines induced by HI contribute to neuronal injury both directly by activating apoptotic mechanisms and indirectly via microglial activation, peripheral leukocyte infiltration, inducing toxic NO and ROS production [88]. Clinical data suggests a strong correlation between raised inflammatory cytokines (IL6, IL8) with severity of injury and poor neurodevelopmental outcomes in term infants with NE [85,89,90]; though it is unclear whether cytokines are themselves pathological or are a reflection of the underlying injurious mechanisms taking place.

Epidemiological data suggests that infection and inflammation exposure render the immature brain vulnerable to HI. The risk of cerebral palsy in preterm infants is increased in the presence of infective risk factors, such as chorioamnionitis (OR 4.2, CI 1.4 - 12), prolonged rupture of membranes (OR 2.3, CI 1.2-4.2) and maternal infection (OR 2.3, CI 1.2 - 4.5) [80]. Antenatal infection combined with a potentially birth-asphyxiating condition dramatically increased the risk of cerebral palsy (OR 78, CI 4.8 - 406) compared to either potentially birth asphyxiating condition (OR 2.5, CI 1.0 – 6.7) or infection (OR 7.2, CI 2.7 - 20) alone [91]. Furthermore, neonatal bacteraemia and funisitis have both been identified as independent risk factors for NE in a resource-limited setting [92].

The close association between perinatal infection, brain damage and neurodisability was demonstrated by Eklind and colleagues. P7 rodents exposed to E. Coli lipopolysaccharide (LPS) 4 hours prior to mild HI, which itself caused little or no injury, developed extensive cerebral volume loss [93]. Our group has demonstrated that LPS administered 4 hours prior to hypoxia dramatically increased neuronal cell death compared to hypoxia or LPS alone [94] (see publication in Appendix A2).

Timing of sensitisation is a significant factor in the evolution of inflammatory mediated brain injury. Inflammation-sensitisation with LPS administrated acutely (4 – 6 hours) or chronically (72 hours) prior to HI exacerbated neuronal cell death in P7 rat pups [95], however when given 24 hours prior to insult was

neuroprotective [93,95,96]. This preconditioning effect was maturity dependent with LPS-sensitisation providing neuroprotection when given up to 96 hours in adult rodents [97–99].

Although neuroinflammation is an important mechanism of brain injury, there are inherent limitations in attributing the presence of specific cytokines or cell lines to histological and MR indicators of brain injury. Selective sampling of specific cytokines provides only a small window into the vast cascade of inflammatory cell signalling that contributes to or attenuates neuronal injury. Publication bias may overestimate the impact of commonly studied cytokines and underestimate the importance of less known regulators of inflammation. A systematic review of inflammatory biomarkers in brain injury is needed to consolidate the evidence to date and establish principal targets to be assessed in pre-clinical and clinical trials in the future.

*1.2.3 Inflammation and therapeutic hypothermia*

Inflammation is a dynamic process and plays an important role in both the evolution of injury as well as subsequent repair and regeneration. Understanding and unpicking this complex cascade is critical to developing methods to modulate the host response to tissue injury.

Jenkins and colleagues examined serum cytokine levels in a cohort of term encephalopathic infants undergoing a clinical trial of hypothermia [100]. Both pro-inflammatory (IL6, 8, MCP1) and anti-inflammatory (IL10) cytokines were elevated in the babies treated with hypothermia. Interestingly, hypothermia

appeared to alter the pattern of cytokine expression with IL6, IL8 and MCP1 demonstrating a secondary peak at 24 – 56 hours unrelated to re-warming. This biphasic pattern was most evident among hypothermic infants with good neurological outcomes. This secondary rise in inflammatory cytokines may indicate the activation of reparative mechanisms, highlighting that pro-inflammatory is not synonymous with pro-injury.

Evaluating serum cytokine profiles in clinical studies have limitations; timing of injury is often uncertain and may precede birth by hours; the severity of HI is variable in depth and duration; and there is an inherent genetic variability in tolerance to hypoxia. The complex inflammatory cascade following perinatal asphyxia is therefore best explored in the preclinical setting where the type and severity of injury may be tightly controlled.

Intra-ischaemic hypothermia in rodents undergoing permanent middle cerebral artery occlusion (MCAO) inhibited the cerebral tissue expression of both anti-inflammatory IL10 and pro-inflammatory cytokines INFc, TNFα, IL2 and IL1β [101]. Rodents treated with hypothermia immediately following transient MCAO occlusion demonstrated reduced leucocyte adhesion molecules (ICAM-1), phagocytic cells (ED-1 positive) and activated microglia [102].

In a piglet study of NE, Rocha-Ferreira and colleagues [103] noted a complex relationship between cytokine levels and hypothermia. Although serum TNFα levels were significantly lower in the hypothermia group compared to normothermia, this was not consistent with other pro-inflammatory cytokines

such as IL1β and IL6. Interestingly, IL10 increased significantly after 36 hours in the normothermia animals but not in those receiving hypothermia. Pro/anti-inflammatory cytokine status was calculated by dividing pro-inflammatory cytokines (IL1β, IL4, 6, 8, TNFα) by an anti-inflammatory cytokine (IL10). Crucially, hypothermia did not suppress the pro-inflammatory cytokine response and in fact, there was a switch to a more pro-inflammatory state in serum cytokines 36 hours after HI in animals receiving hypothermia.

Pre-clinical trials of hypothermic neuroprotection were primarily performed in models of HI. There is therefore limited data exploring whether its efficacy persists in infants exposed to both inflammatory and HI injury. Osredkar and colleagues demonstrated that 5 hours of hypothermia was rendered ineffective in P7 rat pups injected with LPS 4 hours prior to left carotid artery ligation and global hypoxia [104]. The combination of LPS-sensitisation followed by HI exacerbated apoptotic neuronal cell loss, reduced NeuN positive cells (a neuronal biomarker), increased caspase 3 activation, increased astrogliosis and microglial activation. Hypothermia treatment did not appear to attenuate these changes [105]. Consistent with these findings, our group has demonstrated LPS-sensitisation prior to hypoxia in piglets significantly increased cell death compared to either LPS or hypoxia alone [94] and rendered hypothermia ineffective [106].

The influence of inflammation exposure on neurological injury is also pathogen dependent. LPS is a lipopolysaccharide simulating infection by gram-negative organisms, triggering the immune response via Toll-like receptor 4 (TLR-4).

Early onset neonatal sepsis is also commonly caused by gram-positive organisms such as Group B Streptococcus, which initiates the immune response via alternative TLR-2 pathways. Falck and colleagues [107] demonstrated that HI initiated 8 hours following an intraperitoneal injection of PAM3CSK4 (TLR-2 agonist) in P7 rodents also exacerbated neurological injury. In contrast to the LPS studies, hypothermia significantly reduced infarct volume, microglial activation and neuronal survival in the CA1 hippocampus in the PAM3CSK4 exposed animals.

Exacerbation of neurological injury following inflammation-sensitised perinatal asphyxia is a significant concern and hypothermia may not be the most appropriate treatment in these individuals. It is therefore essential we develop methods to identify those infants who have received this 'double-hit' within the 6 hour therapeutic window in order to facilitate patient-specific treatments. At present, there are no biomarkers identified that clearly distinguish hypoxia injury from inflammation-sensitised hypoxia.

### 1.3 Biomarkers of neuronal injury

Establishing biomarkers to stratify the severity of cerebral injury in the hours and days after birth is crucial both in determining the need for therapeutic hypothermia as well as providing accurate information to counsel and support parents. Identifying infants at risk of significant neurological sequelae is essential to initiate early interventions to optimise outcomes. Amplitude-

integrated EEG, MRI, MR spectroscopy and serum brain biomarkers have all been used to stratify severity and prognosticate in infants with NE.

*1.3.1 Amplitude-integrated EEG (aEEG)*

Amplitude-integrated EEG, or cerebral functioning monitoring (CFM) is a spatially and time compressed recording of raw cerebral electrical activity across scalp electrodes. This is plotted on a semi-logarithmic scale in order to better visualise periods of low voltage activity. This provides a cheaper and simpler method to monitor cerebral function in the hours and days following perinatal asphyxia compared to standard EEG. In the pre-cooling era, aEEG patterns in the first 6 hours after birth strongly correlated with long-term outcome [108] and recovery of electrical activity within 24 hours was associated with a favourable prognosis. Following the introduction of therapeutic hypothermia, early aEEG is less predictive and recovery of electrical activity may be delayed [109].

*1.3.2 Near infra-red spectroscopy (NIRS)*

Near infra-red spectroscopy provides continuous bedside monitoring of cerebral haemodynamics and tissue oxygenation. Though not a direct measure of regional cerebral blood flow, NIRS measure regional tissue oxygenation which is a surrogate of the ratio between oxygen supply and demand. Cerebral blood flow measured using arterial spin labelled perfusion MRI and mixed venous saturations strongly correlated with NIRS recorded just prior to and following scans [110]. Broadband NIRS provides additional

measurements of cerebral haemodynamics and metabolism; including oxygenated haemoglobin (HbO2), deoxygenated haemoglobin (HHb) and changes in redox state of cytochrome c oxidase (oxCCO). Cerebral oxygenation (HbD) is calculated from the difference between HbO2 and HHb. Total haemoglobin (HbT) is calculated from the summation of HbO2 and HHb.

Though NIRS has yet to be introduced into routine clinical practice, it remains a useful and informative bedside tool to explore cerebral energetics and support prognostication. A reduction in cerebral metabolism with oxygenation is associated with unfavourable MRS outcomes in infants with NE [111]. Among infants with severe NE, oxCCO strongly correlated with systemic physiology; suggesting that in the severely injured brain there is a lower capacity for CCO to buffer changes in oxygen supply [112].

*1.3.3 Magnetic resonance spectroscopy (MRS)*

Proton MRS provides insight into the metabolic profile of the injured brain within a specified region of interest. The magnetic resonance biomarkers in NE (MARBLE) study [113] recruited 223 infants and assessed the prognostic accuracy of clinical examination, EEG, structural MRI and proton MRS. Thalamic N-acetylcysteine aspartate (NAA), a marker of neuron viability, was the single best predictor of adverse neurological outcome at 2 years (concentration < 5.6 mmol/kg wet weight; sensitivity 100%, specificity 97%). Cerebral lactate increased with progressive injury due to secondary energy failure. The rise in cerebral lactate in association with a fall in NAA (Lac/NAA ratio) has previously been identified as an accurate predictor of adverse

outcomes. In the MARBLE study, the prognostic accuracy was less than expected with a sensitivity of 88% and specificity 90%. Mitra and colleagues [114] however demonstrated Lac/NAA greater than 0.39 in the basal ganglia / thalamus had a sensitivity and specificity of 100% and 97% for adverse 2 year motor outcomes; 90% and 97% for cognitive delay; and 81% and 97% for language delay. The reason for the discrepancy between these two studies is unclear and may reflect differences in the techniques used to calculate the peak area ratios of the metabolites. In addition, the MARBLE study reported a binary outcome of normal vs abnormal neurodevelopment rather than individual domains. Both studies however highlight MRS as a useful prognostic indicator in infants with NE. Adoption into routine clinical practice however remains a challenge due to the lack of availability of 3 Tesla MRI and absence of standardised data acquisition techniques.

*1.3.4 Laboratory biomarkers*

Limitations in the predictive value of clinical examination have led to extensive research into laboratory biomarkers that can accurately and consistently quantify the severity of brain injury and predict long-term prognosis. Unfortunately, there are no serum, urine or CSF investigations that are sufficiently sensitive, specific and practical to be used in routine clinical practice. Candidate biomarkers that have been evaluated to date broadly fall into two categories: inflammatory cytokines and brain-specific proteins.

a. Inflammatory cytokines

Elevated pro-inflammatory cytokines IL1, 6, 8 and TNFα in the first 24 hours following perinatal asphyxia have all been associated with abnormalities on MRI [115], MRS [90] and abnormal neurodevelopment at 12 – 24 months age [89,90,100]. Serum IL6 was associated with injury severity and poor neurodevelopmental outcomes in a cohort of non-cooled [85,90] and cooled [100] infants with HIE. The anti-inflammatory cytokine IL10 has also been associated with adverse neurological outcomes in infants with NE [115] and traumatic brain injury [116]. Based on a review of the literature, a summary of cytokines commonly evaluated in infants with NE is listed in Table 3.

The precise role of cytokines in cerebral injury is unclear and individual cytokines may have both injurious and reparative roles. For example, IL6 increases vascular permeability and can cause cerebral oedema [117]; however it also inhibits TNFα, IL1 and promotes nerve growth factor secretion.

| Cytokine | Function | Evidence in NE |
|---|---|---|
| IL1β | Promotes injury through generation of free radicals, propagating inflammatory reaction, enhancing the toxicity of amino acids. | Increased umbilical cord IL1β predicts neurological abnormalities in HIE after 6-12 months [118]. |
| IL8 | Neutrophil chemotaxis factor, therefore propagating the inflammatory cascade. | Blood and CSF IL1β, IL6 and TNFα were increased in 24 infants with HIE compared with 12 controls. CSF IL1β best predicted abnormal neurology and development at 6 – 12 months [84]. |
| TNFα | TNFα promotes IL1β and IL8 production, induces neuronal apoptosis and disrupts the blood-brain barrier. | Elevated IL1, 6, 8 and TNFα in the first 24 hours has been associated with injury severity [119], abnormal MRI [115], MRS [90] and abnormal neurodevelopment at 12 - 24 months [89,90,100]. |
| IL6 | Pro- and anti-inflammatory properties. Inhibits synthesis of TNFα and IL1β, promotes nerve growth factor secretion. However, can also induce inflammation, increase vascular permeability and secondary oedema. | Umbilical cord IL6 levels were 376-fold higher among infants with HIE and 5.5-fold higher in infants with asphyxia (without HIE) compared to controls [85]. |
| IL10 | Inhibits secretion of IL1β, IL8 and TNFα. Inhibits leukocyte aggregation and attenuates the inflammatory response. | Serum IL6 and IL10 distinguished infants with a favourable compared adverse outcome in a cohort of cooled infants for HIE in the first 24 hours after birth [120]. |
| IL18 | Structurally related to the IL1 family, though reported to have both pro- and anti-inflammatory properties. IL18 is expressed in blood monocytes as well as astrocytes and microglia. | Serum IL18 was elevated in infants with HIE and levels correlate with injury severity [117]. |

*Table 3. Inflammatory cytokines as biomarkers of injury in NE*

b. Brain-specific proteins

Neuron specific enolase (NSE), encoded by the ENO2 (enolase 2) gene, is a glycolytic enzyme found in mature central and peripheral neurons and is released following neuronal and astrocyte cell death. Red blood cells, liver, smooth muscle and lymphocytes also express NSE, though in significantly lower quantities than neuronal cells [117]. Higher NSE levels have been associated with increased mortality and adverse neurological outcomes at 12 months in both the serum [121] and CSF [122] of infants with moderate to severe NE. In a systematic review of biomarkers, elevated CSF NSE in the first 96 hours after birth was an accurate predictor of adverse outcomes [123].

S100B is a neurotrophic calcium-binding protein in astroglia and is a marker of astrocyte cell injury. It has previously been identified as a prognostic biomarker in traumatic brain injury [124] and in small cohorts of normothermic encephalopathic infants in cord blood [125], serum [126] and saliva [127]. In an observational study of cooled infants, significantly higher levels of urinary S100B were present in those infants with an unfavourable outcome (abnormal neurology or abnormal MRI) compared to favourable outcomes (normal neurological examination at discharge) [128]. Though this study was limited by the absence of long-term follow up; a normal neurological examination at discharge does not necessarily translate to normal neurological outcome.

Massaro and colleagues examined the diagnostic and long-term prognostic potential of serum NSE and S100B at serial time points during hypothermia in

infants with moderate to severe NE [129]. Elevated serum S100B and NSE during hypothermia indicated adverse motor and cognitive outcomes after adjusting for baseline and socioeconomic variables. This study had limitations due to a significant proportion of infants lost to follow up; out of 68 infants who survived to discharge, 19 (28%) were lost to follow up. Those lost to follow up represented a higher risk population with a large proportion being recipients of medical assistance and having a lower level of maternal education. Despite the risk of attrition bias, this study highlighted the predictive value of early serum biomarkers in the post-cooling era.

Glial fibrillary acidic protein (GFAP), a cytoskeletal filament protein in astrocytes, has been cited as a promising biomarker in studies of traumatic brain injury [130]. It is found exclusively within the central nervous system and so is highly specific for neuronal cell damage. A raised and persistent serum GFAP was predictive of MRI abnormalities in infants cooled for moderate to severe HIE [131,132] and adverse outcomes at 15 - 18 months [89]. Another candidate biomarker exclusively found in neuronal tissue is ubiquitin carboxyl-terminal hydrolase L1 (UCHL1). Elevated UCHL1 is a sensitive marker of traumatic brain injury and has potential to help stratify which patients require CT scans. In a small cohort of 14 encephalopathic newborns (some of which received therapeutic hypothermia), Douglas-Escobar and colleagues [133] reported significantly higher levels of UCHL1 among infants who died, though the enzyme did not reliably distinguish adverse outcomes among survivors. Serial analysis of UCHL1 after birth suggest levels rise soon after injury with highest concentration in cord blood [89] or at initiation of hypothermia (mean

age 4.4 hours of life) [134]. Enzyme levels fell in the subsequent hours such that by 12 hours, serum concentrations were no longer predictive of abnormal MRI [134] or developmental outcomes at 15 - 18 months. This illustrates the critical importance of timing when interpreting biomarker results.

Tau protein is a cytoskeletal element released during neuronal damage. Elevated serum tau was observed in neonates with bilirubin encephalopathy, correlating with the degree of brain injury severity [135]. In a biomarker study nested within a trial for the use of erythropoietin in HIE, raised serum tau at baseline (within 24 hours of birth) significantly correlated with poor neurodevelopmental outcome at 1 year [132]. This study also investigated brain derived neurotrophic factor (BDNF), a neurotrophin secreted by neurons and astrocytes. Raised BDNF on day 5 was associated with reduced injury on MRI and improved outcomes at 1 year, consistent with its role promoting growth, differentiation and regeneration. Developmental outcomes were measured using a parents' questionnaire score at 1 year. This study was limited by the absence of long term follow up data and potential reporting bias in using parental questionnaires. Both tau and BDNF have been investigated extensively in traumatic brain injuries [136–138], however the evidence in NE is limited and mostly from small observational studies [139–141] .

Several other trophic factors, growth factors and structural proteins have been investigated as potential markers of injury severity and have been summarised in Table 4.

| Name | Role | Location | Example of use in NE |
|------|------|----------|----------------------|
| Glial fibrillary acidic protein (GFAP) | Cytoskeletal protein | Present in astrocytes; CNS specific. | Raised serum GFAP associated with MRI abnormalities [124,125] and adverse outcomes at 15-18 months [79] in infants with moderate to severe NE. |
| Neuron-specific enolase (NSE) | Glycolytic enzyme | Present in mature central and peripheral neurons. Also expressed by red blood cells, liver, smooth muscle and lymphocytes. | Elevated serum NSE predicts adverse motor and cognitive outcomes [123]. |
| S100B | Neurotrophic calcium-binding protein | Primarily present in astrocytes and Schwann cells. Also found in adipocytes, chondrocytes, skin, glioblastoma and melanoma cells. | Elevated serum S100B predicts adverse motor and cognitive outcomes [123]. |
| Ubiquitin carboxy-terminal hydrolase L1 (UCHL1) | Cysteine protease | Predominately expressed in neurons. Small amounts in neuroendocrine cells. | Early marker of injury, rising in cord blood [79] or within hours of birth [127], distinguishing infants with the most severe NE. |
| Tau | Structural protein | Primarily located in axons, forming the axonal microtubule bundles. | Raised tau in the first 24 hours after birth was associated with adverse outcomes at 1 year [132] . |
| Microtubule associated protein 2 (MAP2) | Structural protein | Primarily located in dendrites. | Differential expression of MAP2 in brainstems of children who died from perinatal asphyxia compared to control samples [142]. |

| | | | |
|---|---|---|---|
| Myelin Basic Protein (MBP) | Structural protein | Component of myelin sheath. Abundant in white matter. | Serum MBP in neonates with moderate to severe HIE was significantly higher than mild or no injury [117]. |
| Brain derived growth factor (BDNF) | Neurotrophic factor | Secreted by CNS neurons and astrocytes. | Raised BDNF at day 5 was associated with reduced injury on MRI and improved outcomes at 1 year [132]. |
| Activin A | Neurotrophic factor<br><br>Member of the transforming growth factor β (TGFβ) family. | Produced by several different tissues (pituitary, placenta, gonads). In the CNS, thought to regulate neuron proliferation. | Increased serum Activin A noted in infants with HIE compared to controls. Higher serum concentrations associated with increased severity of injury (infants not cooled) [143]. |
| Vascular Endothelial growth factor (VEGF) | Angiogenic Factor | Secreted by astrocytes and microglia. Neuroprotective properties; promotes proliferation of neuron precursor cells, supports survival and regeneration. | Low serum VEGF on day 1 was associated with moderate to severe HIE and mortality in case control study of infants affected by perinatal asphyxia [144]. |
| Matrix metalloproteinase-9 (MMP-9) | Proteinase | Degrades vascular basement membrane in cerebral vasculature. Contributes to blood brain barrier dysfunction and cerebral oedema. | Higher serum levels associated with increasing severity of HIE [145]. |

*Table 4. Brain-specific proteins as biomarkers of injury in NE*

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c. Micro-ribonucleic acids

Another group of biomarkers that have recently attracted interest are micro-ribonucleic acids (miRNA). These are small non-coding RNA sequences that regulate the expression of genes through incorporation into an RNA-induced silencing complex (RISC) and targeting specific messenger RNAs (mRNA). This prevents the subsequent translation of these mRNAs and therefore downregulates the production of the encoded protein.

MiRNAs are essential in the development of the central nervous system, including dendritic spine formation, neurodevelopment and neurite outgrowth [146,147]. Their abundance in the CNS and stability in serum make them ideal candidates to use as biomarkers for neuronal injury.

Saustad and colleagues [148] evaluated the temporal profile of selected miRNA in piglets after HI. MiRNA-374a significantly increased during hypoxia, remained elevated at 30 minutes and was downregulated by 9.5 hours post-HI. MiRNA-210 was also an early marker of injury, increasing during HI and rapidly normalising by 30 minutes post-HI. This study suggests that these miRNAs have a role in regulating pathological processes during HI.

MiRNA-210 is thought to play a key role in neuro-inflammation following HI. Li and colleagues [149] reported elevated miRNA-210 expression in activated microglia in P7 rat pups as early as 6 hours post-HI. Consistent with these

results, inhibition of miRNA-210 decreased microglial activation, attenuated expression of pro-inflammatory cytokines and reduced cerebral infarct volume.

O'Sullivan and colleagues [150] examined a panel of miRNA in the umbilical cord blood of infants with NE, perinatal asphyxia (without encephalopathy) and controls as part of the Biomarkers in Hypoxic-Ischaemic Encephalopathy (BiHiVE) study. They identified 3 miRNAs that showed consistently altered expression between different study groups; miRNA-374a was significantly reduced in infants with HIE compared to controls; miRNA-376c was reduced in infants with perinatal asphyxia compared to controls; and miRNA-181b was significantly lower in those infants that required hypothermia.

In summary, miRNAs represent a novel group of regulatory biological compounds that have both diagnostic as well as therapeutic potential. Differential expression of specific miRNAs may be used to support clinicians in determining which infants are at most risk of HIE and help stratify those that require therapeutic hypothermia. Dried blood spots have been shown to be a reliable and consistent method of obtaining samples, correlating well with serum [151]. However, whilst certain miRNAs have been associated with specific pathological processes, there are no studies to date examining the differential expression and time course of miRNAs following inflammatory and hypoxic mediated injuries.

## 1.4 Animal models of NE

The history of animal models in the study of perinatal asphyxia extends over two centuries, exploring different patterns of injury generated by HI in both preterm and mature animals. In a primate model of NE, two distinct patterns of cerebral injury were identified – acute total asphyxia and chronic partial asphyxia. Several small and large animal models are currently in use, providing a consistent and reproducible HI in order to assess pathophysiological mechanisms and therapeutic interventions in a well-controlled environment (see publication in Appendix A3). A summary of common animal models of NE is detailed in Table 5.

### 1.4.1 Piglet model of NE

The newborn piglet is a well-established large animal model of perinatal asphyxia. Though the piglet brain is more myelinated than a term human infant, they are comparable in maturity [152]. This model was the first to reproduce the delayed ('secondary') phase of energy failure observed in perinatal asphyxia [23] and subsequently confirmed the efficacy of therapeutic hypothermia [65]. Different methods of reproducing HI have been developed by research groups. HI may be performed by either administering 4 - 6% oxygen with 'passive' ischaemia secondary to hypotension [65]; or combining hypoxia with transient 'active' ischaemia using inflatable carotid occlusion devices [153].

Our group at University College London (UCL) have developed a robust and reproducible HI protocol in the newborn piglet to assess efficacy and safety of neuroprotective interventions. A unique aspect of this model is the use of $^{31}$P MRS and / or broadband NIRS to monitor the reduction in cerebral high energy phosphates (NTP) or cerebral mitochondrial metabolism (oxCCO), respectively. Genetic heterogeneity results in a variable tolerance to hypoxia between individual animals. Our high-resolution method of monitoring physiological and neurometabolic processes during HI enables us to tailor the insult to individual animals and therefore ensure insult severity is standardised. Detailed methods are described in Chapter 3.

There are several advantages in using a large animal model such as the piglet. Firstly, its body size is large enough to perform accurate physiological measurements using similar methods to critically unwell human infants. Secondly, the intensive care provided to the animal is based on current neonatal treatment protocols. Thirdly, the animal size is sufficient to use clinically relevant outcome measures, including aEEG, MRI and MRS. This model of HI provides a platform to facilitate translation of promising neuroprotective interventions from the bench to bedside.

One of the disadvantages of using a large animal model is the cost. The high cost of resources, both technical and personnel, to provide 24 hour intensive care is a limiting factor when designing experiments. This factor is mitigated by using highly refined parameters to standardise insult severity and clinically relevant outcome measurements. The numbers required for each experiment

can therefore be reduced, consistent with the principles of 3 Rs – to reduce, replace and refine experiments to minimise animal usage. Whilst the piglet provides excellent short-term neurometabolic and histological data, long-term behavioural analysis has yet to be well established. In order to assess functional outcomes, further development of the model is required.

| Animal | Description | Advantages | Limitations | Examples of research findings |
|---|---|---|---|---|
| Rodent (Vannucci-Rice) | Unilateral carotid artery ligation and subsequent inhalation of 8% oxygen for 90 minutes (classic model). | Inexpensive; experiments involve large numbers.<br><br>At 10-14 days, CNS maturation comparable to term human infant [154].<br><br>Extensive literature on neurochemistry and behavioural assessment. Short and long-term outcome measurements available. [155,156] | Not suitable for invasive physiological measurements due to small body size [156].<br><br>Rapid postnatal brain maturation; accurate age at experimentation is crucial [154,156].<br><br>White matter varies between species, particularly in early life [157]. | Assessed neuroprotective impact of;<br>-Hypoglycaemia (deleterious)<br>-Hypothermia (very protective)<br>-Hypoxic preconditioning (protective)<br><br>Evaluation of pathological processes during HI [158]. |
| Non-human primate | Acute total asphyxia: Placenta detached at hysterotomy.<br><br>Partial asphyxia: Ventilating with halothane causing hypotension. | Closest phylogenic origin to human [159].<br><br>Body size suitable to perform accurate physiological monitoring.<br><br>Model can include short term physiological, biochemical and long term behavioural outcomes. | Very expensive model.<br><br>Ethical concerns in the use of higher order animal species.<br><br>CNS of baboons and rhesus monkeys are more mature than a human at birth [159]. | Different patterns of injury are associated with the degree and duration of hypoxia/anoxia as well as the presence of acidosis [160]. |

| Piglet | Inhalation of 4-6% oxygen with ischaemia induced passively during hypoxia-induced hypotension or actively with transient carotid artery occlusion. | Well established model with data on cerebral metabolic processes and histology. CNS maturation comparable to human infant [152]. Body size suitable to perform accurate physiological monitoring. | Animals mature rapidly, thus accuracy of postnatal age is important. Severity of HI may be challenging to reproduce consistently [156]. Mostly suited to short term analysis. Long term neurological / behavioural outcome data are not well established. | Cooling ameliorates secondary energy failure [161] with a reduction in excitatory amino acid and nitric oxide release [162]. Cooling reduces degree of apoptosis rather than necrotic cell death [69]. |
|---|---|---|---|---|
| Sheep / Lamb | Several models in use. Exposure to maternal hypoxemia with umbilical cord occlusion at different gestations or bilateral carotid artery occlusion. | HI can be administered on lamb fetus in utero without prior anaesthesia [54,156]. Body size suitable to perform accurate physiological monitoring. Relatively inexpensive. | CNS of lambs are more mature than human newborn [156]. Mostly suited to short term analysis. Long term neurological / behavioural outcome data is not well established. | Secondary energy failure is accompanied by cytotoxic cellular oedema and excitotoxin release [24]. Selective head cooling for 72 hours is safe; reduced cortical infarction and neuronal loss [54]. |

*Table 5. Animal models of NE: advantages and disadvantages*

### 1.5  Magnesium sulphate: a review of neuroprotection

Magnesium is essential to hundreds of enzymatic processes, including energy metabolism, muscle contractility as well as neuronal and neurotransmitter function [163]. Primarily an intracellular cation, magnesium homeostasis is maintained through renal and intestinal absorption and utilisation of storage in bones [163,164]. Serum concentrations are tightly controlled between 0.65 – 1.05 mmol/L (see publication in Appendix A4).

Magnesium was initially used as an anticonvulsant due to its inhibitory effect at neuronal synapses [165].   This anti-excitatory property has led to its extensive research as a neuroprotective agent in several intracerebral pathologies, including cerebral palsy in preterm infants, NE in term infants and ischaemic and haemorrhagic brain injuries in adults.

*1.5.1 Role of magnesium in cellular metabolism*

Magnesium is essential to normal cellular function and has a role in over 300 enzymatic reactions, including glucose utilisation, protein and nucleic acid synthesis [166]. Magnesium competitively antagonises calcium ion entry via post-synaptic N-methyl-D-aspartate (NMDA) receptors (Figure 2) [163]. This depressant effect on synaptic transmission underpins one of the main proposed mechanisms of magnesium neuroprotection.

*1.5.2 Mechanisms of magnesium neuroprotection*

One of the most cited mechanisms of magnesium neuroprotection is its anti-excitotoxic properties, exhibited through NMDA receptor blockade. This post-synaptic receptor plays an important role in the formation of memories through long-term potentiation [167].
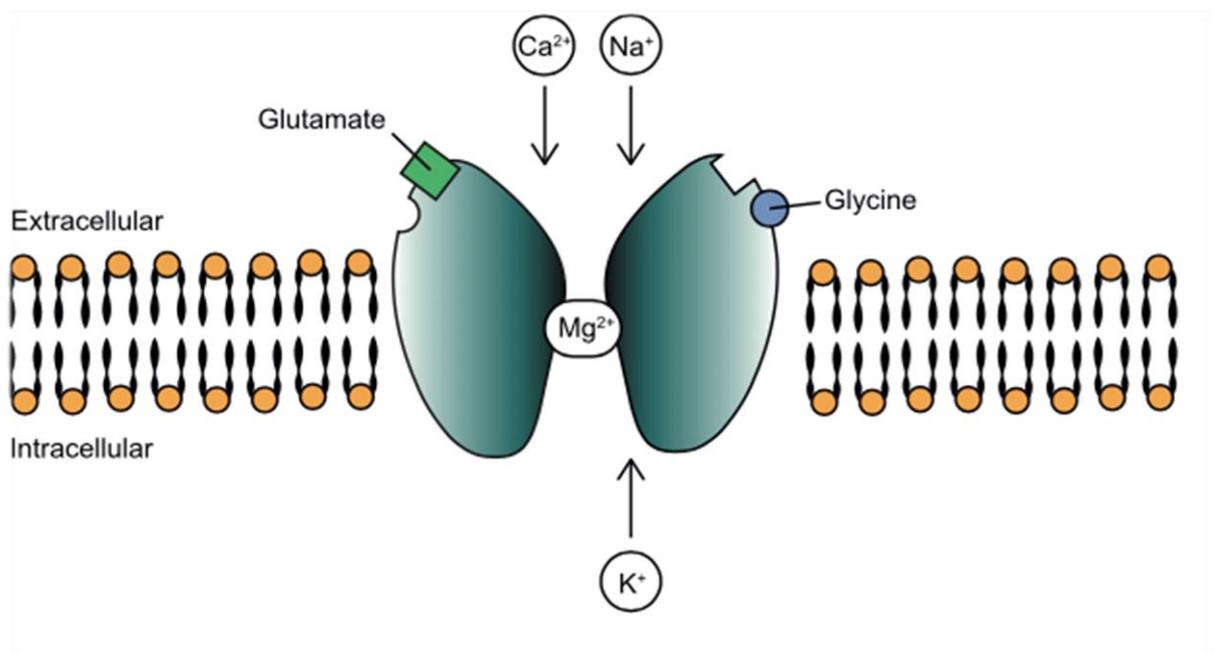


**Figure 2. N-methyl-D-aspartate (NMDA) receptor**

Activation by excitatory amino acids permits the influx of calcium ions, a secondary messenger for physiological cell processes. Excessive calcium ion entry however is pathological and initiates the production of catabolic enzymes (proteases, phospholipases, endonucleases) and free radical production, ultimately leading to apoptosis and necrosis. Inhibition of calcium ion entry by magnesium may prevent this secondary cascade of injury [163]. The anti-

excitotoxic properties of magnesium are well supported by experimental data. $MgSO_4$ exposure attenuated cerebral injury induced by ibotenate, a glutamatergic agonist in mice [168] and reduced extracellular glutamate in gerbils following focal cerebral ischaemia [169].

Magnesium may also be anti-inflammatory. $MgSO_4$ exposure significantly reduced pro-inflammatory cytokine (IL6, TNFα) production in LPS exposed pregnant rodents [170] as well as improved the learning ability in the offspring at 3 months [171]. The mechanism of this anti-inflammatory response is thought to be via inhibition of the Nuclear Factor kB (NF-kB) signal pathway. NF-kB is a transcription factor activated by inflammatory and / or immunological stimuli. Upon activation, NF-kb initiates transcription of genes that increase pro-inflammatory cytokines, adhesion molecules and pro-apoptotic factors [172]. Gao and colleagues (2013) demonstrated that incubating LPS-activated microglia in high concentrations of $MgSO_4$ (above 5 mmol/L) inhibited NF-kB translocation from the cytoplasm to the nucleus in a dose-dependent manner [173]. These findings, however, have yet to be validated in the clinical setting. In a subgroup analysis of the NICHD cohort of women enrolled for preterm neuroprotection, the antenatal administration of $MgSO_4$ did not improve neurodevelopmental outcomes of those infants exposed to chorioamnionitis [174].

### 1.5.3 Evidence of efficacy in animal models of term NE

Pre-clinical evidence supporting magnesium as a neuroprotective agent in term NE are conflicted [175]. Adult rodent studies demonstrating efficacy were confounded by accidental hypothermia and experiments that actively maintained normothermia failed to show benefit. In piglets after HI, $MgSO_4$ as a sole agent has failed to demonstrate a reduction in MRS biomarkers of injury [176] or severity of tissue damage [177]. In addition, $MgSO_4$ did not result in improvement of EEG or neuronal loss in term-equivalent fetal sheep undergoing umbilical cord occlusion [178]. Interestingly adult rodent studies have suggested the combination of $MgSO_4$ and therapeutic hypothermia may be additive [179–181] (see Section 1.5.6).

Prolonged magnesium infusions have been shown to impair oligodendrocyte maturation in preterm fetal sheep [182], highlighting the need for further pre-clinical assessment of both safety and efficacy before translating to human trials.

### 1.5.4 Fetal neuroprotection

Magnesium neuroprotection in preterm infants was first noted by Nelson and Grether (1995) who noted that $MgSO_4$ exposure was lower among very low birth weight infants (VLBW; <1500g) with cerebral palsy compared to VLBW controls (7.1% vs 36%) [183]. The association of *in utero* magnesium exposure and reduced risk of cerebral palsy however was not consistent [184,185] and

there were some concerns of increased mortality in extreme preterm infants [186].

Since then, several large prospective randomised controlled trials have been conducted to assess the safety and efficacy of $MgSO_4$ as a fetal neuroprotective agent (see Table 6). In the Magnesium Endpoint Trial (MagNET 2002) [187], women in threatened preterm labour were recruited between 24 - 34 weeks gestational age and stratified into either a tocolysis ($MgSO_4$ vs other tocolysis) or neuroprotection ($MgSO_4$ vs Saline) study arm. Higher mortality among infants receiving $MgSO_4$ led to early termination of this study. Despite combining the trial arms, $MgSO_4$ did not significantly reduce the risk of cerebral palsy. Two further trials, the Collaborative Trial of Magnesium Sulphate ($ACTOMgSO_4$) [188] and PREMAG [189] demonstrated the safety of $MgSO_4$ therapy, but showed no overall reduction of cerebral palsy at 2 years. In 2008, Rouse and colleagues [190] demonstrated antenatal administration of $MgSO_4$ significantly reduced moderate to severe (GMFCS 2 - 4) cerebral palsy in a large, well designed randomised control trial.

| Study | Inclusion and Exclusion criteria (n=numbers) | Dose & Timing of MgSO$_4$ | Outcome measure | Summary of findings |
|---|---|---|---|---|
| BEAM [190]<br><br>Rouse (2008) | <u>Inclusion:</u><br>GA 24-31 weeks in high risk of preterm delivery within 2-24 hours (PPROM, cervical dilation 4-8cm). (n=2241)<br><br><u>Excluded:</u><br>Delivery likely within 2 hours or dilatation > 8cm, PPROM before 22 weeks, fetal anomalies, maternal contraindication to MgSO$_4$. | BOLUS: 6g<br><br>INFUSION: 2g/h | Composite (death by 1 year or moderate-severe CP by 2 years). | No difference in primary composite outcome (RR 0.97, CI 0.77-1.23).<br><br>Reduction in moderate to severe CP by 2 years (RR 0.55, CI 0.32-0.95). |
| MagNET [187]<br><br>Mittendorf (2002) | <u>Inclusion:</u><br>GA 24-34 weeks in preterm labour<br><br>*1. Tocolysis arm*<br>Active prem labour (dilatation < 4cm); (n=92)<br><br>*2.Neuroprotection*<br>Active prem labour (dilatation > 4cm); (n=57)<br><br><u>Excluded:</u><br>Clinical evidence of pre-eclampsia or infection. | *Tocolysis* BOLUS: 4g<br><br>INFUSION: 2-3g/h<br><br>*Neuro-protection* BOLUS: 4g<br><br>INFUSION: none | Cranial US during admission.<br><br>Diagnosis of CP at 18 months. | Analysis of both groups combined highlighted increased composite adverse outcomes with magnesium (IVH, PVL, CP, death).<br><br>(OR 2.0, CI 0.99-4.1) |
| PREMAG [189]<br><br>Marret (2006) | <u>Inclusion:</u><br>GA < 33 weeks (no lower limit). Expected to delivery within 24 hours; (n=573) | BOLUS: 4g<br><br>INFUSION: none | Neonatal mortality before discharge.<br><br>Severe white matter injury on cranial US. | No significant benefit in mortality or white matter injury. |

| | | | | |
|---|---|---|---|---|
| | Excluded: <br> Fetal malformations, cardiovascular instability, renal impairment, pregnancy associated vascular disease, indication for LSCS, recent ingestion of calcium channel blocker, digitalis, indomethacin, received steroids / aminoglycosides / betamimetics in last 1 hour. | | | |
| ACTO-MgSO$_4$ [188] <br><br> Crowther (2003) | Inclusion: <br> GA < 30 weeks (no lower limit). Birth expected within 24 hours; (n=1062). <br><br> Excluded: <br> Already in 2$^{nd}$ stage of labour, received MgSO$_4$ in this pregnancy, any contraindications to MgSO$_4$ (RR <16/min, absent patella reflex, UO<100ml/4h). | BOLUS: 4g <br><br> INFUSION: 1 g/h | Mortality up to 2 years, CP at 2 years, combined outcome. | No significant difference in mortality (RR 0.83, CI 0.64-1.09), cerebral palsy (RR 0.83, CI 0.66-1.03) or combined outcome (RR 0.75, CI 0.59-0.96). |
| Magpie [191] <br><br> Duley (2002) | Inclusion: <br> Not delivered or within 24 hours post-partum Pre-eclampsia; (n=1544; <37 weeks) <br><br> Excluded: <br> Hypersensitivity to magnesium, renal impairment, myasthenia gravis. | BOLUS: 4g <br><br> INFUSION: 1 g/h | Eclampsia. <br><br> Mortality at discharge. | No clear difference in neonatal mortality (RR 1·02, 99% CI 0·92–1·14). |

***Table 6. Clinical studies of MgSO$_4$ in preterm neuroprotection***

To date, there have been at least six meta-analyses [192–195] and a cost effectiveness evaluation [196] that all support antenatal $MgSO_4$ for preterm neuroprotective. In a meta-analysis of individual participant data [197], magnesium sulphate had a strong protective effect for cerebral palsy in survivors; one case of cerebral palsy was prevented for every 46 mothers receiving magnesium sulphate. This treatment effect varied little by the cause of preterm delivery, gestational age and total dose. Early concerns of high neonatal mortality with magnesium exposure [187] have not been borne out in the subsequent randomised controlled trials and a recent systematic review found no clear association of antenatal magnesium and neonatal harm [198]. The National Institute of Clinical Excellence (NICE) currently recommends $MgSO_4$ in mothers in preterm labour at gestational ages less than 30 weeks and consider use in those between 30 – 33+6 weeks gestation age [199].

Two studies followed up participants into school age. The $ACTOMgSO_4$ study found no significant difference in cognitive, academic, attention or behavioural outcomes at 6 – 11 years [200]. This study was limited by a significant attrition rate (23%), though those lost to follow up had similar maternal, sociodemographic and 2-year outcome variables to those who were assessed. Long-term follow up of the PREMAG cohort (7–14 years) [201] found no significant improvement in neuromotor, cognitive or language ability. This study however had limitations; there were significantly more male infants and a lower mean birth weight in the magnesium group compared to placebo, both of which may have negatively influenced long-term outcomes. Furthermore,

the statistical power of the study was reduced due to the high proportion of infants (almost 30%) lost to follow up.

Therefore, whilst the results from meta-analyses are compelling, ongoing evaluation and surveillance is necessary to determine whether the benefits observed in clinical trials will translate to a population-wide reduction in cerebral palsy.

*1.5.5 Neonatal neuroprotection*

Magnesium is thought to play a critical role in the evolution of cerebral injury following perinatal asphyxia. Low magnesium levels at birth have been observed in infants with severe HIE (0.64 mmol/L, 0.47–0.87) compared to mild or no HIE (0.81 mmol/L, 95% CI 0.75-0.87) and controls (0.72 mmol/L, 95% CI 0.69–0.76) [202]. Though it is unclear whether hypomagnesaemia is a cause or consequence of severe brain injury.

Magnesium has been extensively investigated as a neuroprotective agent in term NE, however these trials have been almost exclusively performed in the pre-cooling era (see Table 7). There was however significant heterogeneity between trials in the timing of intervention, dosing strategies and outcome measures. Although all trials administered $MgSO_4$ within 24 hours of birth, only two initiated treatment within the critical 6 hour therapeutic window [203,204]. One study administered a single 250mg/kg dose $MgSO_4$ [205] while others

used an initial dose of 250mg/kg followed by 125mg/kg [178,204] or 250mg/kg [203,206] at 24 and 48 hours.

Bhat (2009) [203] and Ichiba (2002) [206] both reported improved short-term outcomes, including normal neurology examination, normal CT brain and oral feeding by 2 weeks. However no significant neurodevelopmental benefit was observed at 6 months [204] and 2 years [178]. Ichiba and colleagues repeated their study in 30 newborns with moderate to severe HIE (based on Sarnat criteria) and administered $MgSO_4$ within 6 hours of birth [207]. They reported normal neuro-developmental outcomes in 73% infants at 18 months, though the study was limited by the absence of a control arm.

Kashiba and colleagues reported higher levels of CSF glutamate and aspartate in infants with more severe HIE, supporting excitotoxicity as a primary pathological mechanism of injury. $MgSO_4$ however did not significantly alter levels of these amino acids.

A meta-analysis of these trials indicated $MgSO_4$ reduced the risk of an 'unfavourable' outcome (defined as abnormal neurology, aEEG or neuroimaging) (RR 0.48, 95% CI 0.30-0.77) [208]. However, the small sample sizes, heterogeneous methodology and absence of long-term follow up data significantly limits the validity of these findings.

| Study | Type of study, MgSO$_4$ Dose & Timing (n=numbers) | Inclusion criteria | Outcome measure | Summary of findings |
|---|---|---|---|---|
| Bhat [203]<br><br>(2009) | Single centre RCT (n=40)<br><br>MgSO$_4$<br>250mg/kg/dose daily for 3 days.<br><br>*1st dose given within 3h of birth.* | Age < 6h<br>Gestation > 37 weeks<br>Moderate-Severe HIE.<br><br>Evidence of perinatal asphyxia (3 out of 4):<br>-Signs of fetal distress<br>-IPPV required > 2min<br>-pH <7, BE>15 in first 1h of life<br>-5min Apgar < 6 | Time to oral feeds.<br><br>Neurological exam at discharge.<br><br>CT brain (day 14).<br><br>EEG within 72h and on day 14. | 2 patients had apnoeas during 2$^{nd}$ dose of MgSO$_4$; needing ventilation.<br><br>Composite "good" outcome (normal neurology, CT scan, sucking) better in magnesium group (OR 5.5, CI 1.2-23.6). |
| Gathwala [204]<br><br>(2010) | Single centre RCT (n=40)<br><br>MgSO$_4$<br>250mg/kg loading dose; 125mg/kg at 24 and 48h.<br><br>*1$^{st}$ dose within 30 min of birth.* | Apgar at 5min < 6<br>Gestation > 37 weeks. | CT brain, EEG, neurology at discharge.<br><br>Development at 6 months (Denver II). | Neurology of infants at randomization not stated.<br><br>No significant difference in EEG, CT findings. No difference in development. |
| Groenendaal [178]<br><br>(2002) | Single centre RCT (n=22)<br><br>MgSO$_4$<br>250mg/kg loading dose; 125mg/kg at 24 and 48h.<br><br>*Time of 1$^{st}$ dose not stated.* | Gestation > 37 weeks.<br><br>At least 3 of:<br>-Sign of fetal distress<br>-cord pH < 7.10<br>-need for resus at birth<br>-Apgar at 10min< 5<br>- IPPV at 10min | aEEG scoring<br>-pre-infusion<br>-first 3h<br>-12h<br>-24h<br><br>Follow up at 24 months (Griffiths'). | Study terminated following the death of 2 infants receiving high magnesium doses.<br><br>No significant effect on aEEG or long-term outcome. |

| Ichiba [206]<br><br>(2002) | Multi- centre RCT (n=33)<br><br>MgSO$_4$ 250mg/kg/dose daily for 3 days.<br><br>Dopamine given to magnesium group (5mcg/kg/min).<br><br>*1$^{st}$ dose given within 24h of birth.* | All the criteria below:<br>- Gestation > 37 weeks<br>- Apgar < 7 at 5min<br>-Need for IPPV at 10min<br>- Seizures within 24h | EEG (day 14) CT (day 14) Establishing oral feeds (day 14).<br><br>Composite 'good ' outcome (normal EEG, CT and feeding). | Mean time of 1$^{st}$ dose 10h.<br><br>Composite short term good outcome was significantly improved in magnesium group (p<0.04).<br><br>No difference between individual outcome measures. |
|---|---|---|---|---|
| Khashaba [205]<br><br>(2006) | Single centre RCT (n=47)<br><br>MgSO$_4$ 250mg/kg/dose one dose only.<br><br>*Dose given within 24h of birth.* | Gestation > 37 weeks Apgar at 5min < 3 And / or 1$^{st}$ gasp > 10min after birth.<br><br>Severity of HIE based on neurological exam. | CSF glutamate and aspartate at admission and 72h. | Overall raised glutamate and aspartate with severity of HIE.<br><br>Excitatory amino acids increased after HI and decreased by 72h. MgSO$_4$ did not alter levels of amino acids in CSF. |

**Table 7. Clinical studies of MgSO$_4$ in term NE**

*1.5.6 Magnesium as an adjunct to hypothermia*

Zhu and colleagues [179] evaluated the efficacy of different durations of mild hypothermia (35$^o$C) in combination with MgSO$_4$, administered either prior to or 2 hours post-global cerebral ischaemia in adult rodents. Normothermia was maintained during HI and animals were sacrificed at 7 days following injury. When administered prior to injury, MgSO$_4$ in combination with 6 hours

hypothermia was significantly more effective than either treatment alone. When interventions were initiated 2 hours post-asphyxia, $MgSO_4$ plus 24 hours of hypothermia was more effective than hypothermia alone. Shorter durations of hypothermia (6 or 12 hours) appeared ineffective with or without magnesium therapy. This study highlights the critical importance of early interventions to maximise benefit.

Similar findings were reported by Campbell and colleagues, who noted a reduction in mean infarct volume at 48 hours in rodents treated with $MgSO_4$ and 24 hours mild hypothermia (35°C) following focal cerebral ischaemia. Interestingly, the efficacy of moderate hypothermia (33°C) was abolished when combined with $MgSO_4$ treatment. The reason for this reduction in efficacy is unclear. The animals used were spontaneously hypertensive rats and therefore $MgSO_4$ may have caused exaggerated cardiovascular compromise. Physiological parameters were only reported during insult and not throughout the experiment. The study does however raise the possibility that $MgSO_4$ combined with hypothermia may in fact be detrimental and therefore it is critical that this relationship be further explored in a pre-clinical setting prior to translation into clinical trials.

Of note, there has been one clinical study examining the benefit of $MgSO_4$ with therapeutic hypothermia in term infants with NE [209]. Reassuringly, the authors reported a favourable safety profile with no difference in death or hypotension between treatment groups. The study however had several methodological limitations, including variable inclusion criteria based on the

availability of EEG, variable methods of cooling (5/60 infants underwent selective head cooling) and lack of reported physiological data. Long-term outcomes for this study have yet to be published.

*1.5.7 Adult neuroprotection*

$MgSO_4$ has also been trialled in adult neurological injuries. In addition to attenuating excitotoxic mediated injury, magnesium may improve reperfusion following a cerebrovascular accident by dilating penetrating cerebral arterioles.

*Acute ischaemic strokes*

In a large, double-blind randomised control trial, adults with a clinical diagnosis of stroke were allocated either magnesium or placebo within 12 hours of symptom onset [210]. Disappointingly, magnesium did not reduce death or disability at 90 days post-event. The lack of efficacy was attributed to the delay in receiving magnesium as only 3% of study participants received the drug within 3 hours of symptom onset. A follow up study by Saver and colleagues (2015) subsequently enrolled 1700 patients to receive magnesium or placebo within 2 hours [211]. Patients received a $MgSO_4$ loading dose by paramedics and commenced a 24-hour maintenance infusion on arrival to hospital. Despite earlier intervention, $MgSO_4$ did not reduce death or disability at 90 days. Final diagnosis of the cohort was primarily acute ischaemic strokes (73%) and then intracranial haemorrhage (23%).

*Haemorrhagic Strokes*

Magnesium has also been trialled in the management of aneurysmal subarachnoid haemorrhage (SAH). Delayed cerebral ischaemia affects almost a third of patients following SAH, exacerbating the existing injury and contributing to poor neurological outcomes [212]. Magnesium did not improve clinical outcomes after aneurysmal SAH in a large randomised control trial [213] or meta-analysis [214].

*1.5.8 Target serum magnesium for neuroprotection*

Experimental data from *in vitro* and *in vivo* small animal models suggest that serum magnesium requires increasing to at least twice baseline levels to achieve neuroprotection, approximately 1.5 – 3 mmol/L [215,216]. Rapidly achieving and maintaining supra-physiological serum concentrations whilst avoiding systemic side effects remains a challenge.

Symptoms of toxicity are dose-dependent and usually start when serum magnesium is greater than 3 mmol/L, increasing in severity at higher concentrations. Initial signs include hypotension, loss of the patella reflex (3.5 – 5 mmol/L), followed by respiratory paralysis (5 - 6.5 mmol/L), altered cardiac conduction (above 7.5mmol/L) and subsequently cardiac arrest (above 12.5 mmol/L) [217].

Magnesium sulphate dosing in previous animal [175] and term infant studies [203,205,206] have mostly involved repeated bolus injections. Penrice and

colleagues (1996) studied the effect of 400mg/kg given 1 hour post-insult followed by 200mg/kg at 12 and 24 hours, however found significant hypotension following administration of the second dose. This bolus regimen resulted in adequate peak serum levels (1.91 - 2.12mmol/L), however this rapidly fell to below target concentrations in-between doses [176]. Similar variations in peak and trough magnesium were seen in the neonatal trials using smaller doses of 250mg/kg daily for 3 days [206] (see Figure 3).
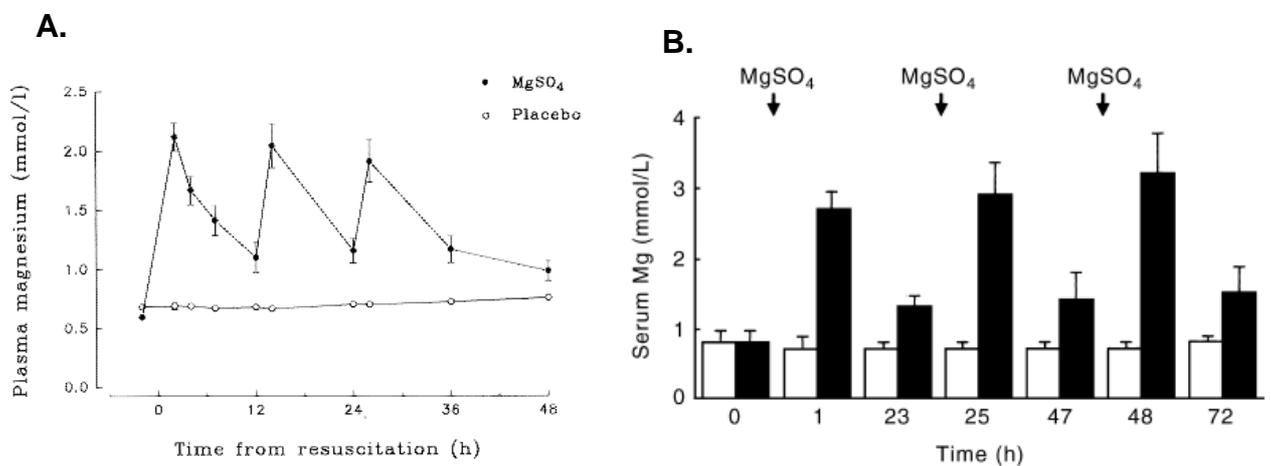


*Figure 3. Serum magnesium following bolus administration in (A) piglet study: MgSO₄ 400mg/kg loading dose; 200mg/kg at 12 and 24 hours; (B) human study: MgSO₄ 250mg/kg daily (■ ) versus placebo (□ )*

Administering $MgSO_4$ as a loading dose and continuous infusion provides a more stable method to achieve supra-physiological serum magnesium. Galinsky and colleagues achieved a steady raised serum magnesium level (1.72 - 1.89mmol/L) using a 160mg loading dose and 48mg/hour infusion in preterm fetal sheep [182].

Magnesium clearance is primarily renal [166] and may be impaired by both hypoxic kidney injury and poor renal perfusion during hypothermia [218]. There is limited data on serum and CSF magnesium pharmacokinetics in cooled infants following perinatal asphyxia.

*1.5.9 Summary*

Magnesium sulphate is an attractive neuroprotective agent due to its widespread availability, low cost and good safety profile. The anti-excitatory and anti-inflammatory mechanisms are biologically plausible and supported by pre-clinical data.

The trials of $MgSO_4$ in perinatal asphyxia are insufficient to either advocate or refute its use in term NE. Given the well documented benefits of hypothermic neuroprotection, future studies of $MgSO_4$ would now have be as an adjunctive rather than standalone therapy. Exploring the safety and efficacy of $MgSO_4$ in combination with hypothermia in a large animal model of NE represents the next step in the translational pathway to clinical practice

## Chapter 2


## Hypotheses

***Hypothesis 1: MgSO₄ in combination with moderate hypothermia is more effective than hypothermia alone***

Magnesium sulphate is thought to attenuate excitotoxic injury through NMDA receptor blockade of calcium ions into neurons post-hypoxia. Hypothermia has also been shown to protect against toxic calcium influx through altered AMPA subunit expression. The combination of $MgSO_4$ and hypothermia may therefore increase the neuroprotective efficacy beyond hypothermia alone. This hypothesis is supported by data from adult rodent studies where $MgSO_4$ administered within 2 hours following index HI combined with 24 hours of mild hypothermia was more effective than hypothermia alone [179,180].

This study aims to (i) establish a safe $MgSO_4$ dosing regimen to maintain suprasystemic serum magnesium over 48 hours, and (ii) to determine the safety and efficacy of $MgSO_4$ in combination with hypothermia in a clinically translatable piglet model of term NE.

***Hypothesis 2: MgSO₄ is anti-inflammatory and alters the gene expression of pro-inflammatory cytokines***

Inflammation is well recognised as a contributor to the progressive neuronal injury that persists in the hours, days and weeks following perinatal asphyxia. This is particularly relevant in the preterm population, where exposure to maternal chorioamnionitis significantly increases the risk of periventricular leukomalacia and cerebral palsy [219]. Experimental data suggests

magnesium is anti-inflammatory; *in vivo* MgSO$_4$ exposure reduced TNFα and IL6 production in maternal monocytes [220]; and magnesium-treated pregnant rodents exposed to inflammatory stimuli (LPS) demonstrated reduced IL6 and TNFα production [170] with improved learning ability in the offspring [171]. The ability to modulate the inflammatory response may in part explain why antenatal magnesium administration has been successful in reducing the risk of cerebral palsy in preterm infants.

We aim to evaluate whether MgSO$_4$ exposure alters the gene expression of inflammatory cytokines and brain biomarkers in a cohort of piglets undergoing hypothermic neuroprotection following HI.

***Hypothesis 3: Serum biomarkers can identify the infants with NE with prior exposure to infection and inflammation***

Although it is well established that inflammation exacerbates brain injury following perinatal asphyxia, there are limited tools available to identify encephalopathic infants with a predominately hypoxic injury from those with inflammation-sensitised hypoxia. Clinical examination is insufficient and early infection markers are insensitive. Infants with inflammation-sensitised hypoxia may be more responsive to specific anti-inflammatory therapies and agents such as MgSO$_4$ may prove particularly efficacious. Therefore, in addition to searching for additional neuroprotective agents, it is essential we also develop biomarkers to help differentiate injury phenotypes.

We aim to (i) evaluate a panel of inflammatory and brain-specific serum biomarkers in hypoxia (Hypoxia), inflammation-sensitisation (LPS) and combined LPS and hypoxia (LPS + Hypoxia) in a piglet model of NE, and (ii) to establish whether these biomarkers correlate with the severity of cerebral injury.

**Chapter 3:**


**Methods**

### 3.1 Ethics

Experimental and surgical procedures were performed in accordance with UK Home Office Guidelines [Animals (scientific procedures) Act, 1986] and UCL Animal Welfare and Ethical Review Body (AWERB). The study complies with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

### 3.2 Anaesthesia and surgical preparation

Large white piglets aged under 36 hours and weighing between 1.7 to 2.1 kg were initially sedated with an intramuscular midazolam injection (0.2 mg/kg) and subsequently anesthetised with isoflurane mixed with air (3% v/v during surgery, 1.5 - 2.5% during experimentation).

Only male animals were included in this study in order to reduce variability between groups and minimise group sizes in accordance with the 3R's principle; replace, reduce and refine study design to minimise the use of animals during experimentation.

Animals remained insentient throughout experimentation. Following insertion of a tracheostomy, animals were mechanically ventilated (SLE 2000 infant ventilator, Surrey, UK) to maintain $PaO_2$ 8 - 13 kPa and $pCO_2$ 4.5 - 6.5 kPa. Remotely inflatable carotid occluders (OC2A, In Vivo Metric) were carefully sewn around the common carotid arteries. An umbilical arterial line was inserted for invasive mean arterial blood pressure (MABP) and heart rate (HR)

monitoring. The arterial line was infused with heparinised saline (0.5 IU/ml in 0.9% sodium chloride) at 0.3 ml/hr. An umbilical venous catheter and / or peripherally inserted central venous catheter (PICC) line was inserted for infusion administration. Infusions included maintenance 10% dextrose at 60 ml/kg/day (reduced to 40 ml/kg/d post-insult), fentanyl 3-6 mcg/kg/h and antibiotics (benzylpenicillin 50mg/kg/dose BD, gentamicin 5mg/kg/dose OD).

Animals were nursed prone in a stereotactic frame built within a purpose-built MR compatible transport incubator. Intensive care support was provided for the animal throughout the experiment by an experienced technician and neonatologist.

Surgical procedures, anaesthetic agents and methods of euthanasia are all known to effect cell death. We have previously performed naive (animals sacrificed prior to experimentation) and sham experiments (surgery and 48 hours of anaesthesia without HI or study intervention). Although some cell death was observed in sham experiments, this was not significantly different from naive controls and markedly less than animals that had received HI [94].

### 3.3 Treatment of complications

Hypotension: Persistent hypotension following insult was treated with a 0.9% saline bolus (Baxter; 10ml/kg) up to a maximum of 20 ml/kg in 24 hours and inotropic support. Infusions of inotropes included dopamine (5-20 μg/kg/min),

dobutamine (5-20 µg/kg/min) and noradrenaline (20-500 ng/kg/min) added sequentially and doses titrated to maintain MABP > 40 mmHg.

Hyperkalaemia: Serum potassium levels above 7 mmol/L were treated with salbutamol boluses (4 µg/kg) and 10% calcium gluconate.

Seizures: Clinical seizures were defined as rhythmical abnormal movements of the limbs that were clearly distinguishable from shivering. Electrical seizures were defined as a sudden rise and narrowing of the integrated aEEG trace with associated repetitive stereotyped activity on the raw EEG. Seizures were treated with a loading dose of phenobarbitone 20 mg/kg with a second loading dose (10 mg/kg) if required.

Of note, isoflurane anaesthesia is anti-epileptogenic. This likely reduces the overall seizure burden in piglets following HI. As animals all receive the same depth of anaesthesia, we anticipated this would not significantly impact our ability to assess the efficacy of the study intervention. The use of isoflurane anaesthesia may however result in an underestimation of the brain injury associated with seizures that would be observed in the clinical setting.

### 3.4 Hypoxia ischaemia protocol

#### 3.4.1 Hypoxia with transient carotid artery occlusion

(Performed in Part (I): The safety and efficacy of $MgSO_4$ in combination with moderate hypothermia)

Animals were monitored for at least 1 hour following surgery to ensure cardiovascular parameters, EEG and baseline broadband NIRS were normal prior to HI. The insult was initiated by simultaneous reduction of fraction of inspired oxygen ($FiO_2$) and inflation of the carotid occluders. $FiO_2$ was decreased stepwise to 6% over the first 3 minutes and titrated to changes in the MABP, broadband NIRS and EEG activity. Oxygen delivery was liberalised in the event of a mean BP < 27 mmHg or 3-fold decrease in brain tissue oxidation of cytochrome c oxidase (oxCCO) and restricted further if recovery of EEG was observed during insult. Blood gas analysis was performed at 5 minute intervals and the fall in pH, BE and rise in lactate were recorded.

Total duration of HI was anticipated to be 20 – 25 minutes, depending on the duration of isoelectric EEG, hypotension (MABP < 30 mmHg), total fall in oxCCO (AUC oxCCO), total reduction in $FiO_2$ (AUC $FiO_2$) and blood gas analysis. Two experienced team members decided on the duration of the insult using the available information. At the end of the insult the animal was resuscitated, occluders deflated and $FiO_2$ increased to 21%. The duration of the HI was typically 20 – 25 minutes (see Figure 4).
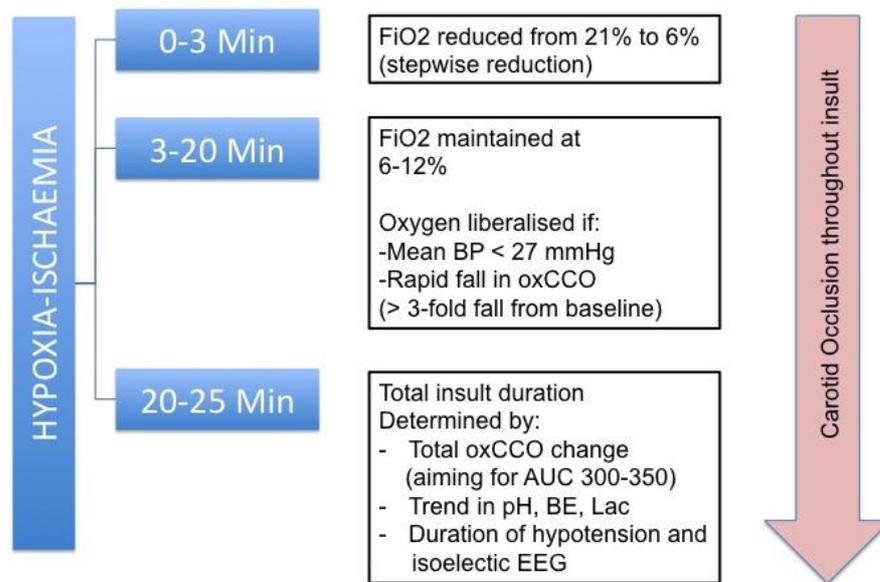
**Figure 4. Hypoxia ischaemia protocol using transient carotid occlusion**

*3.4.2 Hypoxia with hypotension-induced ischaemia*

(Performed in Part (III): Differentiating biomarkers of inflammation and hypoxia brain injury)

Hypoxia and LPS + Hypoxia piglets underwent a global hypoxia insult. LPS was administered 4 hours prior to insult in the LPS + Hypoxia group. Inspired oxygen ($FiO_2$) was reduced in a stepwise manner to 4% over 3 minutes and held for 10 minutes. This was followed by a further 10 – 30 minutes of $FiO_2$ 6 - 12% titrated to standardised MABP parameters. Targeted parameters for the insult included MABP between 26 – 30 mmHg. Overall duration of insult was guided by real-time measurements of the duration of isoelectric EEG (< 5 µV) and hypotension (MABP < 30 mmHg). The insult was terminated early if cardiac arrest appeared imminent as indicated by profound hypotension (< 25 mmHg) or bradycardia.

In the event of a cardiac arrest, the piglet was resuscitated with oxygen, chest compressions and intravenous adrenaline (0.1 ml/kg 1:10000) every 3 - 4 minutes. On return of circulation, the oxygen was reduced to 21% and post-resuscitation care continued. Blood gas analysis and biochemistry was performed immediately after insult to monitor lactate and metabolic acidosis.

### 3.5 Neurological monitoring during HI

#### 3.5.1 Broadband near-infrared spectroscopy

Broadband near-infrared spectroscopy (NIRS; Mini-CYRIL, Cytochrome Research Instrument and Application System) was used to measure cerebral haemodynamics and metabolism in real time, including relative changes in the concentration of oxyhaemoglobin (HbO2), deoxyhaemoglobin (HHb) and oxidised cytochrome c oxidase (oxCCO). Changes from baseline oxCCO during HI provided insight into the degree of brain tissue metabolic suppression and severity of HI [20]. The real-time and total fall in oxCCO during HI measured using the area under the curve below baseline (AUC oxCCO) was used as an indicator of insult severity [21] (Figure 5).

**A.**



**B.**
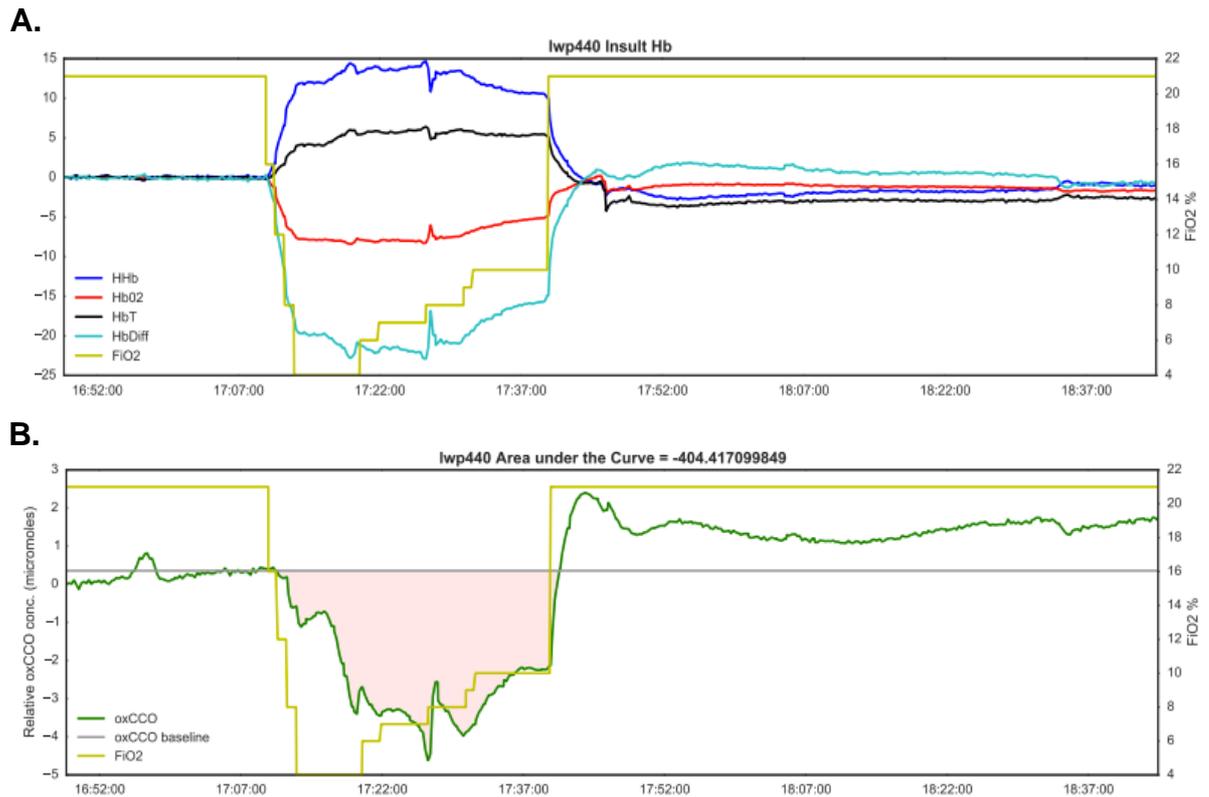


***Figure 5. Near-infrared spectroscopy measuring changes in the concentration of (A) oxyhaemoglobin (HbO2), deoxyhaemoglobin (HHb) and cerebral blood volume (B) oxidisation of cytochrome c oxidase***

*3.5.2 Electro-encephalogram (EEG) and Amplitude-integrated EEG*

A 6-channel EEG (Nicolet) was acquired at baseline, during HI and throughout the experiment (removed only during MRI scans). Both the raw EEG and amplitude-integrated trace were evaluated throughout insult to ensure suppression of cerebral electrical activity. In the event of a recovery of electrical activity during insult, the $FiO_2$ was reduced accordingly.

A mild HI was defined *a priori* as recovery of electrical activity within 1 hour of insult (Figure 6). Animals with mild HI were included in the overall analysis, though a separate subgroup analysis of animals with moderate to severe HI was planned.
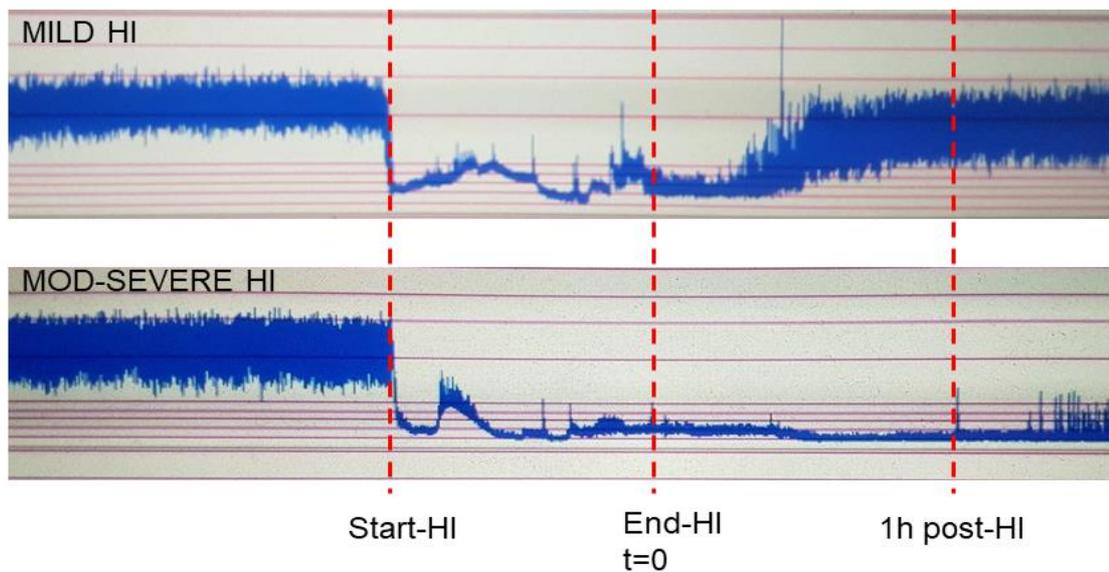


**Figure 6. aEEG recordings at baseline, during and immediately post-HI. Mild HI was defined as recovery of cerebral electrical activity within 1 hour of insult.**

## 3.6 Outcome measurements

### 3.6.1 Magnetic resonance spectroscopy

MRS was performed at 24 and 48 hours in a Philips clinical 3T MRI scanner. ^{31}P MRS spectra was acquired and analysed using the Advanced Method for Accurate, Robust and Efficient Spectral (AMARES) fitting of MRS data [18] as implemented in the jMRUI software. NTP is predominantly composed of

adenosine triphosphate (ATP) and therefore changes in this signal reflect levels of this high-energy phosphate molecule. Measurements of inorganic phosphate ($P_i$), phosphocreatine (PCr), exchangeable phosphate pool (epp = $P_i$ + PCr + 2γ-NTP + β-NTP) were acquired over the whole brain and peak area ratios calculated ($P_i$/epp, PCr/epp, and NTP/epp).

$^1$H MRS spectra was also acquired; voxels were applied by an experienced MR physicist using standardised anatomical landmarks. A white matter voxel was placed in the dorsal right subcortical region at the level of the centrum semiovale (8x8x15mm) and deep grey matter voxel (15x15x10mm) in the thalamus. Data was analysed using jMRUI software and the lactate / N-acetyl aspartate (Lac/NAA) peak area ratio calculated. Comparisons of voxel location with macroscopic neuroanatomy allowed spatial correlation of MRS biomarkers with regional immunohistochemistry (see Figure 8).

*3.6.2 Amplitude-integrated electroencephalogram scoring*

The aEEG analysis and scoring was performed every 15 minutes during the experiment and reviewed independently by two clinicians blinded to the treatment allocation. The scores were averaged in 6 hourly time epochs and compared between groups.

Pattern classification was used as defined by Hellstrom-Westas et al (1995) [19]; isoelectric (0), continuous low voltage (1), burst suppression (2), discontinuous normal voltage (3) and continuous normal voltage (4) (Figure 7).
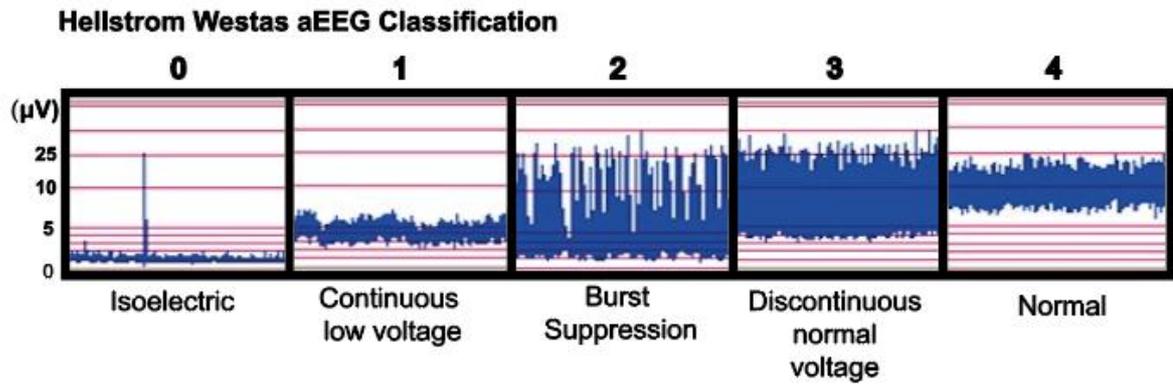
**Figure 7. Hellström-Westas classification of aEEG voltage patterns**

*3.6.3 Immunohistochemistry*

Piglets were euthanised at 48 hours post-HI (intravenous pentobarbital) and fixed with an intra-cardiac injection with cold 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS). The brain was then removed and fixed in 4% PFA for 7 days. The right cerebral hemisphere was dissected in 5 mm coronal slices starting from the anterior optic chiasm and embedded in paraffin wax before being sectioned into 5 μm slides. Two sections per piglet (R0 and R1) were stained (bregma 00 and −2.0) and 8 brain regions were examined; cingulate cortex, sensorimotor cortex, hippocampus, internal capsule, periventricular white matter, caudate, putamen and thalamus (Figure 8). For each animal, 2 sections placed 5mm apart were assessed for each stain. The cerebellum was not assessed for immunohistochemistry.

For all histochemical and immunohistochemical stains, brain sections were dehydrated in xylene (3 × 10 minutes) and rehydrated in graded ethanol solutions (100–70%), followed by double-distilled water.
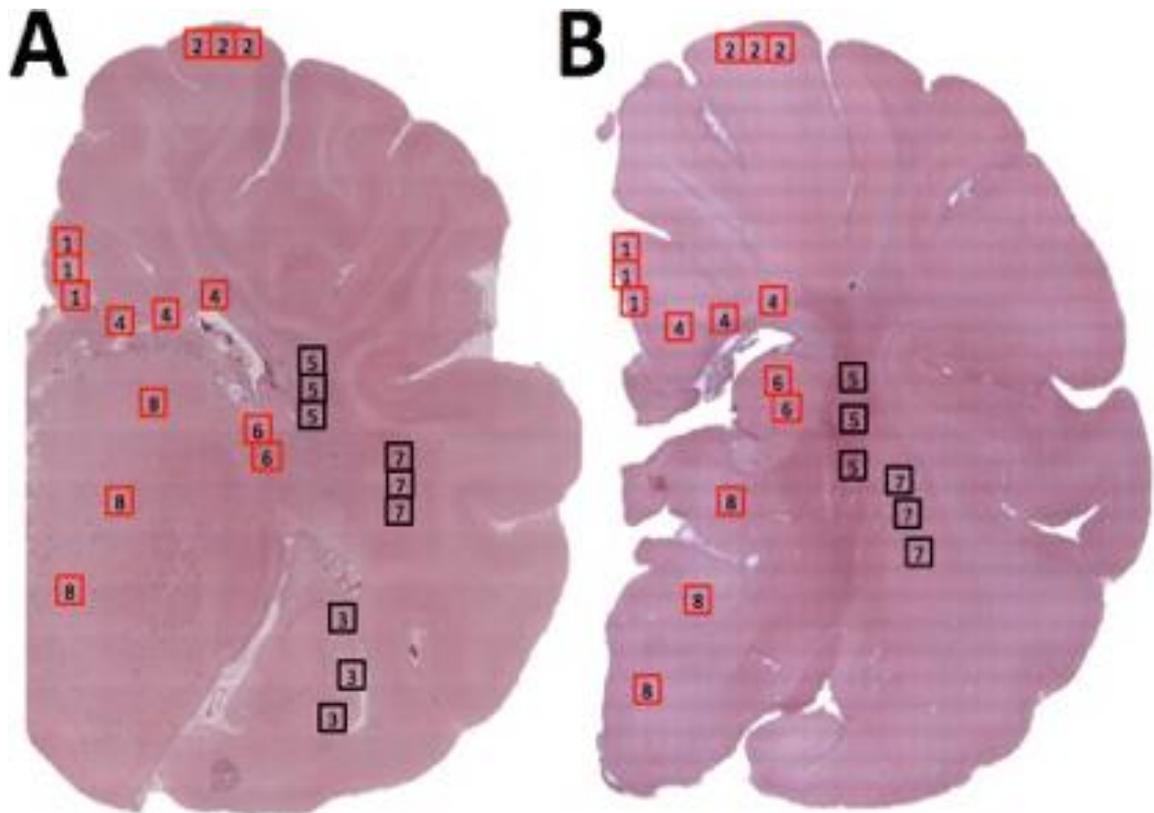


**Brain regions sampled for immunohistochemistry:**

**Included in MRS Voxel:**
(1) Cingulate cortex
(2) Sensory motor cortex
(4) Periventricular white matter
(6) Caudate
(8) Thalamus

**Not Included in MRS Voxel:**
(3) Hippocampus
(5) Internal Capsule
(7) Putamen

*Figure 8. Representative piglet photomicrograph indicating two brain levels (A and B) assessed for immunohistochemistry.*

TUNEL

To assess cell death, sections were stained for nuclear DNA fragmentation using histochemistry with terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL). Endogenous peroxidases were removed by pre-treating sections in 3% $H_2O_2$ in methanol, followed by 15 minute peptidase pre-digestion with 20 µg/ml proteinase K (Promega) at 65°C. Sections were incubated at 37°C for 2 hours with the TUNEL solution (Roche), containing biotinylated dUTP. TUNEL positive cells were counted in 3 fields at x40 magnification (area 0.066 mm$^2$) and averaged per region (cells / mm$^2$).

Caspase 3, Iba1 and OLIG2

Apoptosis and glial activation were assessed by quantifying activated caspase 3 (CC3), glial fibrillary acidic protein (GFAP) and microglial ionised calcium-binding adaptor molecule (Iba1) immunoreactivity. Oligodendrocytes were stained with oligodendrocyte transcription factor (OLIG2) and used as a surrogate marker of myelination.

For activated caspase 3, Iba1 and OLIG2; pre-treatment with Ventana CC1 (950-124), equivalent to EDTA buffer was used. For GFAP, Protease 1 (0.38 mg/mL alkaline protease enzyme activity) was used. Primary antibody incubation was performed with a rabbit antibody against activated Caspase 3 (1:100, Cell Signalling 9661L) for 32 minutes, Iba1 (1:250, WAKO 019-19741) for 4 hours, GFAP (1:1000, DAKO Z0334) for 32 minutes and OLIG2 (1:100, Millipore AB9610) for 4 hours. Incubation with a secondary swine anti-rabbit

immunoglobulin (DAKO E0343) was performed for 44 minutes for activated caspase 3; 1 hour for Iba1 and OLIG2; and for 32 minutes in GFAP staining.

The biotin residues were detected with the avidin-biotinylated horseradish peroxidase complex (ABC, Vector Laboratories) and visualised with diaminobenzidine/$H_2O_2$ (Sigma), with $CoCl_2$ and $NiCl_2$ included to intensify TUNEL histochemistry. The sections were dehydrated in graded alcohol and xylene and mounted with Depex (VWR), or alternatively, mounted with Vectashield + 4',6-diamidino-2-phenylindole (DAPI) aqueous mounting media (Vector Labs), to facilitate total cell number counts during analysis of Iba1 and activated caspase 3.

Iba1 positive microglial cells were assigned a ramification index based on their body and branch density. A 0.049 x 0.049mm square grid (x40 magnification) was placed in three fields for each brain region and the microglial ramification index was calculated as $B^2/C$, where B was the average number of branches crossing the 3 horizontal and vertical gridlines and C was the number of cell bodies within the grid. Activated caspase 3 immunoreactive cells were counted in 3 fields at x20 magnification (area 0.164 $mm^2$) and averaged per region (counts / $mm^2$). Astroglia activation was quantified by measuring GFAP immunoreactivity optical luminosity. The mean brightness was measured in 2 fields per region at x20 magnification and was deducted from the mean brightness of a blank slide [221]. OLIG2 positive cells were counted in 3 fields (x40 magnification; area 0.066 $mm^2$) and averaged per brain region (cells / $mm^2$).

### 3.7 Statistical analysis

Statistical analysis was performed using Prism version 6.0 for Mac, GraphPad Software, La Jolla California USA and JMP®, Version 14, SAS Institute Inc., Cary, NC, 1989-2019. Full statistical analysis is described in the individual methods (Sections 4.2.5, 5.1.6 and 6.1.7). Unless otherwise stated, all analysis used a significance threshold of 0.05.

# Chapter 4

## Part (I): Safety and efficacy of MgSO$_4$ in combination with moderate hypothermia

### 4.1 Preliminary pharmacokinetics study

(Full experimental methods are detailed in Chapter 3).

#### 4.1.1 Magnesium sulphate dilution / administration

The magnesium stock solution was obtained at 50% concentration: 1 gram = 4 mmol (2 ml). The loading dose was diluted to a 5% solution with normal saline and administered 2 hours following HI. The infusion solution was made up to 50 ml with 10% dextrose and calculated to run at 0.5 ml/hr to deliver 10 mg/kg/hour.

#### 4.1.2 Target Serum Concentration

As described in Section 1.5.8, *in vitro* and *in vivo* data suggests increasing magnesium to at least twice baseline levels are necessary to achieve neuroprotection [215,216], though systemic toxicity is a risk when levels exceed 3 mmol/L. Normal serum concentrations of magnesium are 0.7-1.0mmol/L and in order balance efficacy and safety, we opted for a target serum concentration of 1.4 – 2.0 mmol/L.

#### 4.1.3 Study groups

Group 1 (n = 3) piglets received 40 mg/kg $MgSO_4$ loading dose and 10 mg/kg/hour infusion over 48 hours. One animal underwent HI with hypothermia; one animal had HI without hypothermia; and one sham (no HI). Results from these experiments informed dosing regimens of the following group.

Group 2 (n = 5) animals received an increased 80 mg/kg $MgSO_4$ loading dose with variable infusion rates 10 – 20 mg/kg/hour over 12 hours. This was undertaken to determine the effectiveness of an increased bolus dose and higher infusion rates. In total, 4 animals received HI (3 animals underwent hypothermia) and 1 sham.

Group 3 (n = 4) animals received an escalating regimen of loading doses from 120 – 160 mg/kg, followed by infusion rates ranging from 5 – 8 mg/kg/hour. All animals received hypothermia following HI.

*4.1.4 Results from preliminary pharmacokinetics*

*Group 1: $MgSO_4$ bolus 40 mg/kg and Infusion 10 mg/kg/hr*

The $MgSO_4$ bolus of 40 mg/kg did not significantly increase serum magnesium; median baseline serum magnesium increased from 0.85 mmol/L (range 0.77 - 1.09 mmol/L) to 1.0 mmol/L (range 0.94 - 1.09 mmol/L) at 2 hours post-bolus. The 10mg/kg/hour infusion resulted in a slow and steady increase in serum magnesium; however target concentration was only achieved after 24 hours (Figure 9).
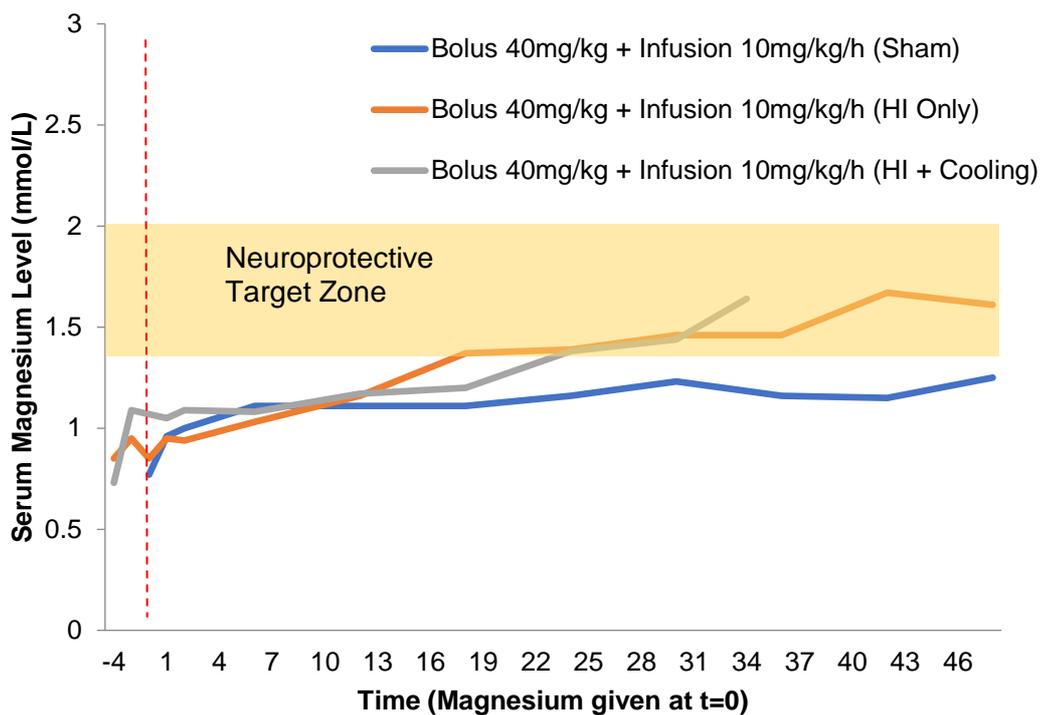
***Figure 9. Group 1 serum magnesium levels: MgSO₄ Bolus 40 mg/kg and infusion 10 mg/kg/hour (treatment initiated at t=0).***

**Conclusion**: MgSO₄ bolus of 40 mg/kg was insufficient to achieve target serum concentrations.

*Group 2: MgSO₄ bolus 80 mg/kg and infusion 10 – 20 mg/kg/hour*

All five animals had similar baseline serum magnesium levels between 0.9 – 1.1 mmol/L. Following a MgSO₄ bolus 80 mg/kg, median serum magnesium increased to 1.4 mmol/L at 1 hour (range 1.1 - 1.6 mmol/L) and 1.3 mmol/L at 2 hours (range 1.3 - 1.6 mmol/L). Therefore, 3 out of 5 animals did not reach target serum levels by 2 hours after the bolus dose. Serum magnesium rapidly accumulated in animals who underwent HI and received infusion rates between 15 – 20 mg/kg/hour (Figure 10).

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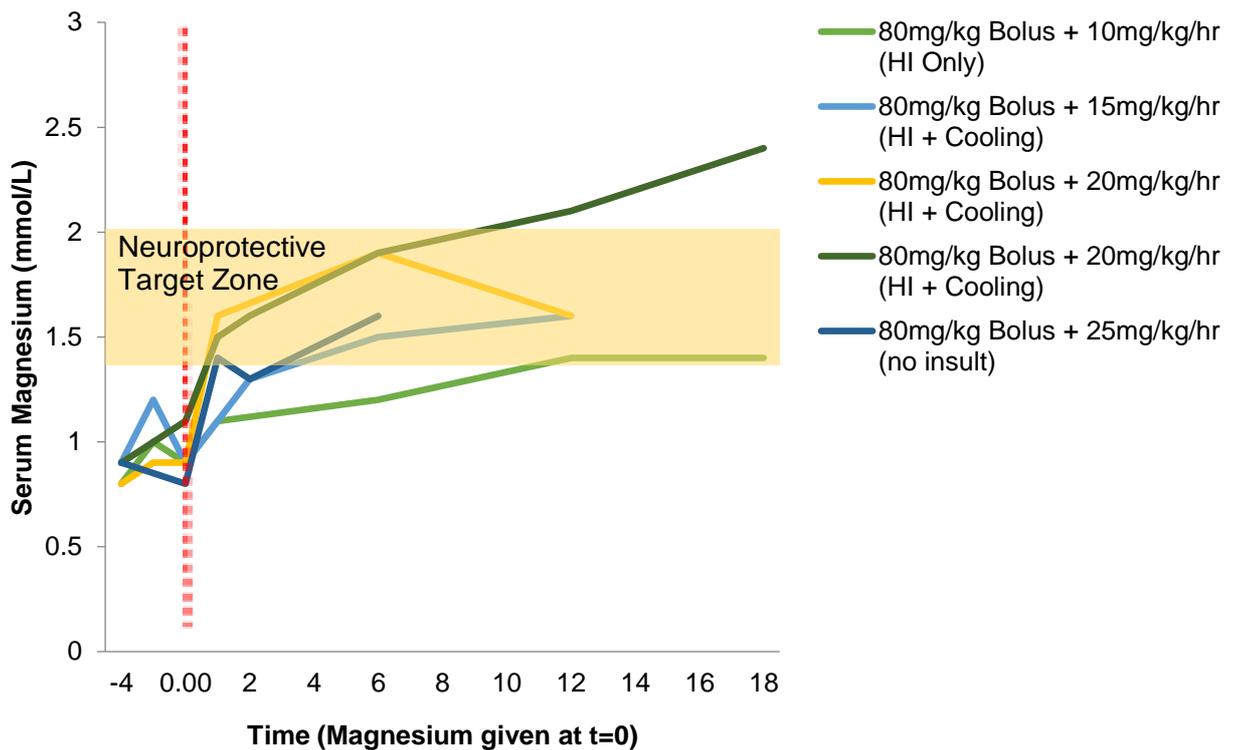
*Figure 10. Group 2 serum magnesium levels: MgSO₄ Bolus 80mg/kg and infusions 10-20mg/kg/hour (treatment initiated at t=0).*

**Conclusion**: MgSO₄ loading dose of 80 mg/kg was insufficient to increase serum magnesium to target concentration within 1 hour. Accumulation of serum magnesium was a significant risk in infusion rates greater than 10 mg/kg/h.

_Group 3: MgSO₄ bolus 120 – 160 mg/kg and Infusions 5 – 8 mg/kg/h_

All four animals within this group underwent HI and were cooled for 24 hours. Animals in successive experiments received an escalating bolus dose, initially MgSO₄ 120 mg/kg, subsequently 160 mg/kg. Following a bolus of 120 mg/kg, serum magnesium increased from 0.77 mmol/L at baseline to 1.25 mmol/L at 1 hour. Using a MgSO₄ bolus dose of 160 mg/kg, target serum concentrations

was achieved at 1 hour; increasing from a median baseline of 0.85 mmol/L (range 0.72 - 0.94 mmol/L) to 1.79 mmol/L (range 1.67 - 1.83 mmol/L) at 1 hour.

Infusion rates between 5 - 7.5 mg/kg/h provided a stable serum magnesium concentration in the first 24 hours, however trended below 1.5 mmol/L after approximately 36 hours. An infusion rate of 8 mg/kg/h resulted in a stable supra-systemic serum magnesium level in one animal. Unfortunately, there was a protocol violation in one animal where the $MgSO_4$ infusion 8 mg/kg/h was not started for 8 hours following the bolus dose (Figure 11).
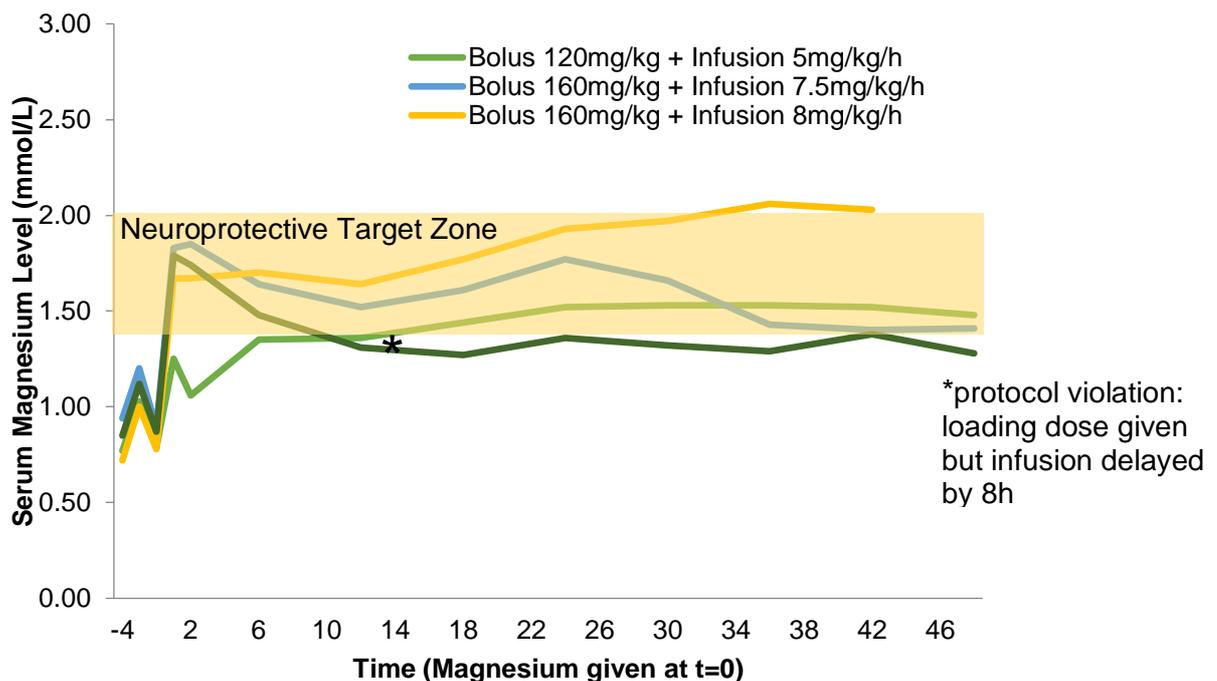


*Figure 11. Group 3 serum magnesium levels: $MgSO_4$ Bolus 120-160 mg/kg and infusions 5-8 mg/kg/hour (treatment initiated at t = 0).*

**Conclusion**: $MgSO_4$ 160 mg/kg bolus rapidly increased serum magnesium concentrations two-fold within 2 hours of administration. This dose achieved

the lower end of the target concentration (1.4 – 2.0 mmol/L). The loading dose was therefore optimised to 180 mg/kg and commenced 1 hour post-HI for the safety and efficacy study of $MgSO_4$ in combination with hypothermia (Section 4.2). Infusion rates at 8 mg/kg/h maintained a stable serum concentration in piglets with HI undergoing therapeutic hypothermia.

### 4.1.5 Survival

During this phase of the experiment, two animals died prior to initiation of treatment (one animal died during surgery and one animal had a cardiac arrest during HI and did not respond to resuscitation).

### 4.1.6 Correlating serum and cerebrospinal fluid magnesium levels

CSF was acquired at baseline and 48 hours in all 9 animals (Figure 12). CSF magnesium levels increased from median baseline 1.03 mmol/L (0.96 - 1.11 mmol/L) to 1.19 mmol/L (1.08 - 1.24 mmol/L). Within individual animals, increases in serum magnesium resulted in a modest rise in CSF levels.
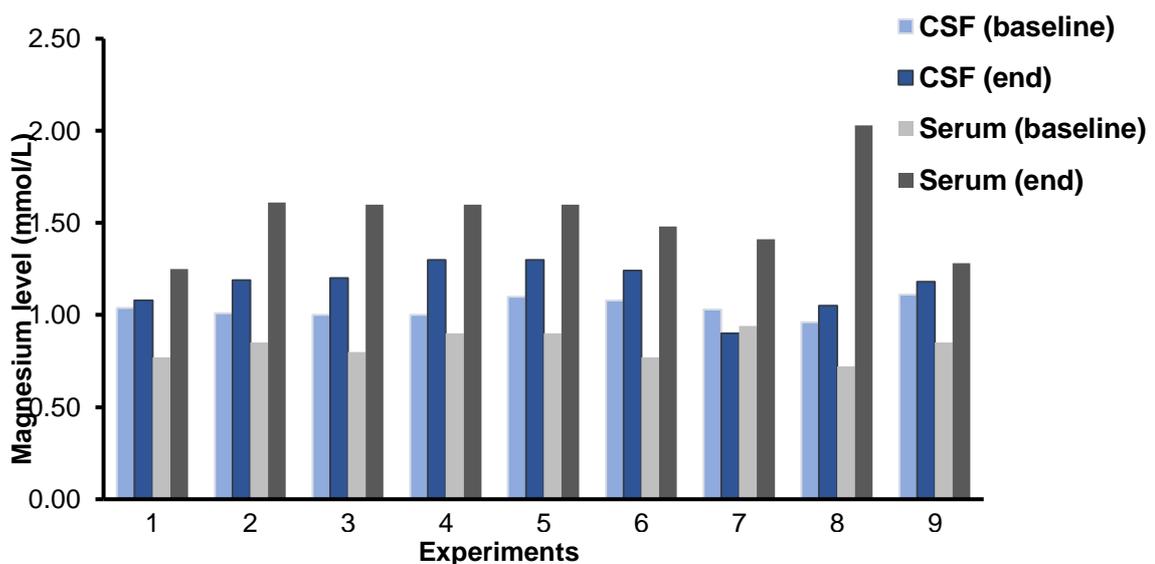


**Figure 12. Serum and CSF magnesium levels at baseline and after 48 hour infusion in individual piglets (pharmacokinetic study).**

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### 4.2 Safety and efficacy of MgSO$_4$ and moderate hypothermia

This study has now been published (see publication- Appendix A5).

#### 4.2.1 Study dose

Based on the preliminary pharmacokinetic results, MgSO$_4$ was administered as 180 mg/kg bolus and 8 mg/kg/h infusion.

#### 4.2.2 Method of HI

Hypoxia with ischaemia via transient carotid artery occlusion.

#### 4.2.3 Study groups and protocol

Block randomisation of 15 animals was computer generated, sealed in opaque envelopes and opened on completion of HI. Animals were allocated to either MgSO$_4$ plus hypothermia (Mg + HT; n=8) or 0.9% saline plus hypothermia (HT, n=7).

Hypothermia was initiated at 1 hour post-insult; whole body therapeutic hypothermia to 33.5$^o$C was maintained for 12 hours. MgSO$_4$ 180 mg/kg bolus was administered at 1 hour post-insult, followed immediately with 8mg/kg/h infusion over 48 hours (Figure 13).
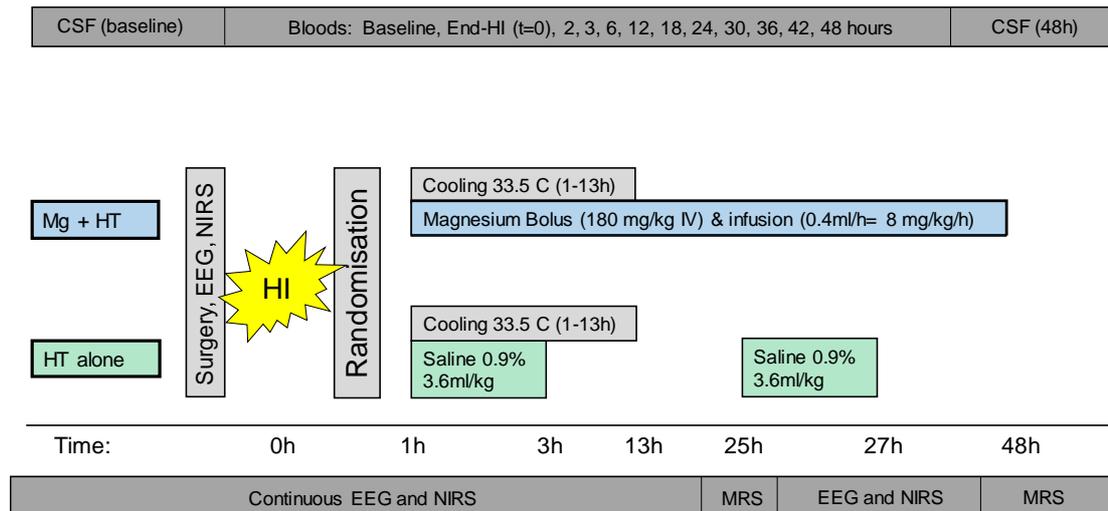
*Figure 13. Study timeline (I): 15 piglets randomised post-HI to either (i) Mg + HT (n=8) or (ii) HT alone (n=7). Animals euthanised at 48 hours.*

### 4.2.4 Blood tests

Point of care blood gas analysis and biochemistry was performed regularly throughout experimentation, including pH, pCO2, pO2, bicarbonate ($HCO_3$), base excess (BE), lactate (Lac), glucose (Glu), urea and electrolytes (Abbot Laboratories, UK). Serum magnesium was taken at baseline, end of insult (t = 0) and 2, 3, 6, 12, 18, 24, 30, 36, 42 and 48 hours post-HI. CSF magnesium was measured at baseline and 48 hours. Serum and cerebrospinal fluid (CSF) magnesium samples were frozen (-20°C) and processed on completion of the study (Royal Veterinary College, Hawkshead, UK).

### 4.2.5 Statistical analysis

Parametric physiological data was analysed using a Student T-test and non-parametric data with Mann-Whitney U test.

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Histology data was $log_{10}$ transformed when results were significantly skewed in order to facilitate parametrical statistical analysis. An ANOVA model was fitted to the mean results for the two brain sections (R0 and R1) with two replicates per subject. Differences between treatment least square means (with 95% confidence intervals) were analysed for each brain region and overall. No adjustments were made for repeated measurements.

Hourly aEEG scores were averaged for each subject into 6 hourly time epochs and an ANOVA model was fitted to include terms for treatment, time interval and treatment by time interval interaction. A random LWP subject effect was also included. The difference between mean scores were analysed between treatment groups at individual time points and overall. The presence of seizures (either clinical or electrical) were noted within an hourly EEG experimental record. The number of hourly time epochs with seizures were compared between study groups.

MRS biomarker results were $log_{10}$ transformed due to the skewed data. Data was fitted to an ANOVA model, including terms for treatment, day of scan (24 or 48 hours) and treatment by day interaction. A random subject effect was also included. Least square mean differences in MRS biomarkers were determined between study groups at 24 and 48 hours and overall.

A post-hoc sensitivity analysis was planned, determining if exclusion of animals with milder injury (defined *a prior* as EEG recovery within 1 hour of HI) altered the primary outcome (MRS Lac/NAA) and differences in EEG scores.

Sample size calculation based on previous piglet studies [153] indicated that MRS biomarkers (Lac/NAA) over 48 hours varied by 1.0U between study groups (standard deviation 0.75) on a log scale. Using a significance threshold of 5% and 80% power, we determined 7 subjects were needed in each group.

*4.2.6 Physiological measurements*

All animals appeared healthy on arrival to the laboratory and mean weight was similar between study groups (2.04 kg vs 2.06 kg, p = 0.84). Baseline cardiovascular parameters (HR, MABP) and biochemistry (Na+, K+, urea, creatinine, glucose) were stable prior to insult with no statistical differences between groups. Animals in the Mg + HT group had a slightly higher baseline $HCO_3$ and BE, though all values were still within the normal range [222] **(Table 8).**

Normothermia (38 - 39°C) was maintained throughout HI. There was no significant difference between HR, MABP and temperature post-insult (0 - 1 hour), during induction of hypothermia (1 – 3 hours), hypothermia (3 – 13 hours), rewarming (13 – 23 hours) and normothermia (23 – 48 hours). Animals in the HT group had a slightly raised HR compared to Mg + HT group on day 2, though still within normal limits [223].

|  | Mg + HT | HT | p-value |
|---|---|---|---|
| Number of piglets (n) | 8 | 7 | |
| Body Weight (kg) | 2.04 (0.15) | 2.06 (0.10) | 0.84 |
| Heart Rate (min$^{-1}$) | | | |
| Baseline | 169 (22.3) | 181 (14.2) | 0.16 |
| End-HI – 1h | 207 (22.0) | 213 (14.2) | 0.67 |
| 1-3h (induction of cooling) | 170 (20.6) | 179 (30.5) | 0.45 |
| 3-13h (cooling) | 169 (12.4) | 181 (15.9) | 0.1 |
| 13-23h (rewarm) | 190 (14.4) | 197 (24.9) | 0.8 |
| 23-48h | 179 (22.2) | 205 (19.9) | **0.03*** |
| MABP (mmHg) | | | |
| Baseline | 52 (4.6) | 53 (7.7) | 0.63 |
| End-HI – 1h | 45 (8.7) | 45 (8.7) | 0.97 |
| 1-3h (induction of cooling) | 43 (3.0) | 42 (3.5) | 0.88 |
| 3-13h (cooling) | 44 (3.3) | 47 (5.5) | 0.32 |
| 13-23h (rewarm) | 46 (8.6) | 45 (5.2) | 0.59 |
| 23-48h | 51 (2.3) | 54 (4.4) | 0.21 |
| Rectal Temperature (ºC) | | | |
| Baseline | 38 (0.8) | 38.5 (0.6) | 0.32 |
| End-HI – 1h | 38.4 (0.5) | 38.7 (0.4) | 0.13 |
| 1-3h (induction of cooling) | 35.6 (1.1) | 36 (1.4) | 0.53 |
| 3-13h (cooling) | 33.7 (0.2) | 33.6 (0.1) | 0.85 |
| 13-23h (rewarm) | 36.2 (0.2) | 36.1 (0.2) | 0.29 |
| 23-48h | 38.4 (0.1) | 38.4 (0.1) | 0.99 |
| pH | | | |
| Baseline | 7.454 (0.07) | 7.404 (0.09) | 0.52 |
| End of insult (time 0) | 7.223 (0.10) | 7.170 (0.05) | 0.22 |
| 12h after time 0 | 7.502 (0.05) | 7.514 (0.05) | 0.65 |
| 24h after time 0 | 7.428 (0.09) | 7.334 (0.07) | 0.07 |
| 48h after time 0 | 7.478 (0.06) | 7.466 (0.06) | 0.74 |
| pCO$_2$ (kPa) | | | |
| Baseline | 5.7 (0.7) | 5.7 (0.9) | 0.88 |
| End of insult (time 0) | 6.0 (1.1) | 5.6 (0.6) | 0.36 |
| 12h after time 0 | 5.2 (0.5) | 4.7 (0.3) | 0.09 |
| 24h after time 0 | 6.0 (0.9) | 7.0 (0.7) | 0.06 |
| 48h after time 0 | 4.9 (0.5) | 5.0 (1.0) | 0.93 |
| pO2 (kPa) | | | |
| Baseline | 16.3 (7.5) | 11.6 (3.2) | 0.16 |
| End of insult (time 0) | 5.9 (3.1) | 6.9 (2.8) | 0.52 |
| 12h after time 0 | 14.6 (7.0) | 11.7 (1.1) | 0.29 |

| | | | |
|---|---|---|---|
| 24h after time 0 | 12.5 (2.1) | 11.5 (3.2) | 0.51 |
| 48h after time 0 | 12.6 (0.7) | 13.8 (2.0) | 0.13 |
| HCO$_3$ (mmol/L) | | | |
| Baseline | 30.1 (2.1) | 26.5 (3.4) | **0.03*** |
| End of insult (time 0) | 18.5 (4.1) | 15.1 (2.5) | 0.09 |
| 12h after time 0 | 31.3 (2.2) | 29.5 (2.2) | 0.16 |
| 24h after time 0 | 29.6 (5.2) | 27.7 (3.6) | 0.50 |
| 48h after time 0 | 27.2 (3.3) | 26.6 (1.5) | 0.72 |
| Base Excess | | | |
| Baseline | 6.5 (2.8) | 2.0 (3.9) | **0.02*** |
| End of insult (time 0) | -9.0 (5.2) | -12.9 (3.4) | 0.12 |
| 12h after time 0 | 7.3 (2.4) | 6.0 (2.9) | 0.39 |
| 24h after time 0 | 5.6 (6.2) | 2.2 (4.4) | 0.31 |
| 48h after time 0 | 4.1 (4.3) | 3.3 (1.5) | 0.68 |
| Lactate | | | |
| Baseline | 4.0 (1.2) | 5.0 (1.9) | 0.25 |
| End of insult (time 0) | 13.5 (3.1) | 14.5 (1.2) | 0.43 |
| 12h after time 0 | 3.2 (1.5) | 3.4 (1.3) | 0.84 |
| 24h after time 0 | 3.2 (2.4) | 3.3 (2.3) | 0.93 |
| 48h after time 0 | 1.2 (0.4) | 1.2 (0.4) | 0.79 |
| Glucose | | | |
| Baseline | 5.7 (0.6) | 6.5 (1.3) | 0.16 |
| End of insult (time 0) | 9.0 (1.8) | 8.9 (3.3) | 0.91 |
| 12h after time 0 | 13.8 (3.8) | 14.7 (3.6) | 0.65 |
| 24h after time 0 | 14.6 (11.9) | 15.3 (12.0) | 0.91 |
| 48h after time 0 | 4.7 (1.8) | 6.9 (2.9) | 0.11 |

*Table 8. Physiological data and blood gas analysis (I), (mean, SD)*

### 4.2.7 Hypoxia-ischaemia insult

All animals underwent a standardised HI delivered by hypoxia and transient carotid artery occlusion. There was no significant difference in the duration of HI, hypotension, isoelectric EEG, AUC oxCCO, AUC FiO$_2$ and post-HI blood gas analysis between study groups indicating that a similar severity of injury was delivered to all piglets (Table 9).

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|  | Mg + HT | HT | p-value |
|---|---|---|---|
| Duration of HI (min) | 23.1 (3.3) | 22.1 (2.7) | 0.39 |
| Duration of Hypotension BP < 30mmHg (min) | 8.5 (3.7) | 8.4 (4.6) | 0.8 |
| End of insult gas | | | |
| pH | 7.223 (0.10) | 7.170 (0.05) | 0.22 |
| BE | -9.0 (5.2) | -12.9 (3.4) | 0.12 |
| Lactate | 13.5 (3.1) | 14.5 (1.2) | 0.43 |
| Duration of EEG <5uV (min) | 19.3 (3.1) | 18.9 (1.3) | 0.87 |
| AUC FiO2 (%) | 310 (63) | 296 (44) | 0.59 |
| AUC oxCCO (ummol) | 255 (108) | 296 (92) | 0.46 |
| Severity of HI | | | |
| -Mild* | 1/8 | 1/7 | 1.0 |
| -Moderate-Severe | 7/8 | 6/7 | |
| *defined as aEEG recovery within 1h of HI | | | |

***Table 9. Hypoxia ischaemia insult parameters (I), (mean, SD)***

We did not observe significant hypotension or increase in inotrope requirements during hypothermia or rewarming / normothermia in animals treated with $MgSO_4$ (Table 10).

One piglet, assigned to the HT group, was euthanised prior to experiment completion at 21 hours post-HI due to intractable hypotension and rising lactate. This animal was subsequently diagnosed with a bowel perforation on post-mortem. All other animals survived to experiment completion. There was no significant difference in survival between Mg + HT and HT groups (8/8 vs 6/7, p = 0.47).

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|  | Mg + HT | HT | p-value |
|---|---|---|---|
| Saline Bolus (ml/kg) | | | |
| 0-13h | 0 | 0 | n/a |
| 13-24h | 0 | 1.4 (3.8) | 0.47 |
| 24-48h | 0 | 0 | n/a |
| Dopamine (mcg/kg/min) | | | |
| 0-13h | 10.8 (2.5) | 11.3 (3.1) | 0.6 |
| 13-24h | 17.8 (2.7) | 19.3 (5.8) | 0.51 |
| 24-48h | 12.2 (8.2) | 12.8 (10.2) | 0.72 |
| Dobutamine (mcg/kg/min) | | | |
| 0-13h | 0.3 (0.5) | 0.1 (0.2) | 0.43 |
| 13-24h | 7.7 (9.7) | 3.9 (7.6) | 0.52 |
| 24-48h | 2.3 (2.5) | 2.9 (7.1) | 0.24 |
| Noradrenaline (ng/kg/min) | | | |
| 0-13h | 0 (0) | 0 (0) | n/a |
| 13-24h | 66.6 (101) | 26.2 (75.0) | 0.49 |
| 24-48h | 14 (30.6) | 4.3 (12.3) | 0.50 |
| Adrenaline (ng/kg/min) | | | |
| 0-13h | 0 (0) | 0 (0) | n/a |
| 13-24h | 35.5 (100) | 114 (302) | 0.73 |
| 24-48h | 0 (0) | 192 (508) | 0.47 |

*Table 10. Volume and inotrope requirements (I), (mean, SD)*

*4.2.8 Serum and CSF pharmacokinetics*

Serum magnesium increased significantly within 1 hour post-$MgSO_4$ bolus (0.72 vs 1.62 mmol/L, p = 0.008). Suprasystemic magnesium concentrations were maintained during the infusion and were unaffected by HT (Figure 14).

Following completion of 5 animals, an interim analysis of magnesium treated animals demonstrated a gradual fall in serum magnesium after 30 hours, trending towards 1.4 mmol/L (the lower end of the intended target range). The infusion dose was therefore increased from 8 mg/kg/h to 10 mg/kg/h for the

remaining 3 animals in the study. CSF magnesium at 48 hours increased significantly from baseline (1.04 vs 1.21 mmol/L, p = 0.008).
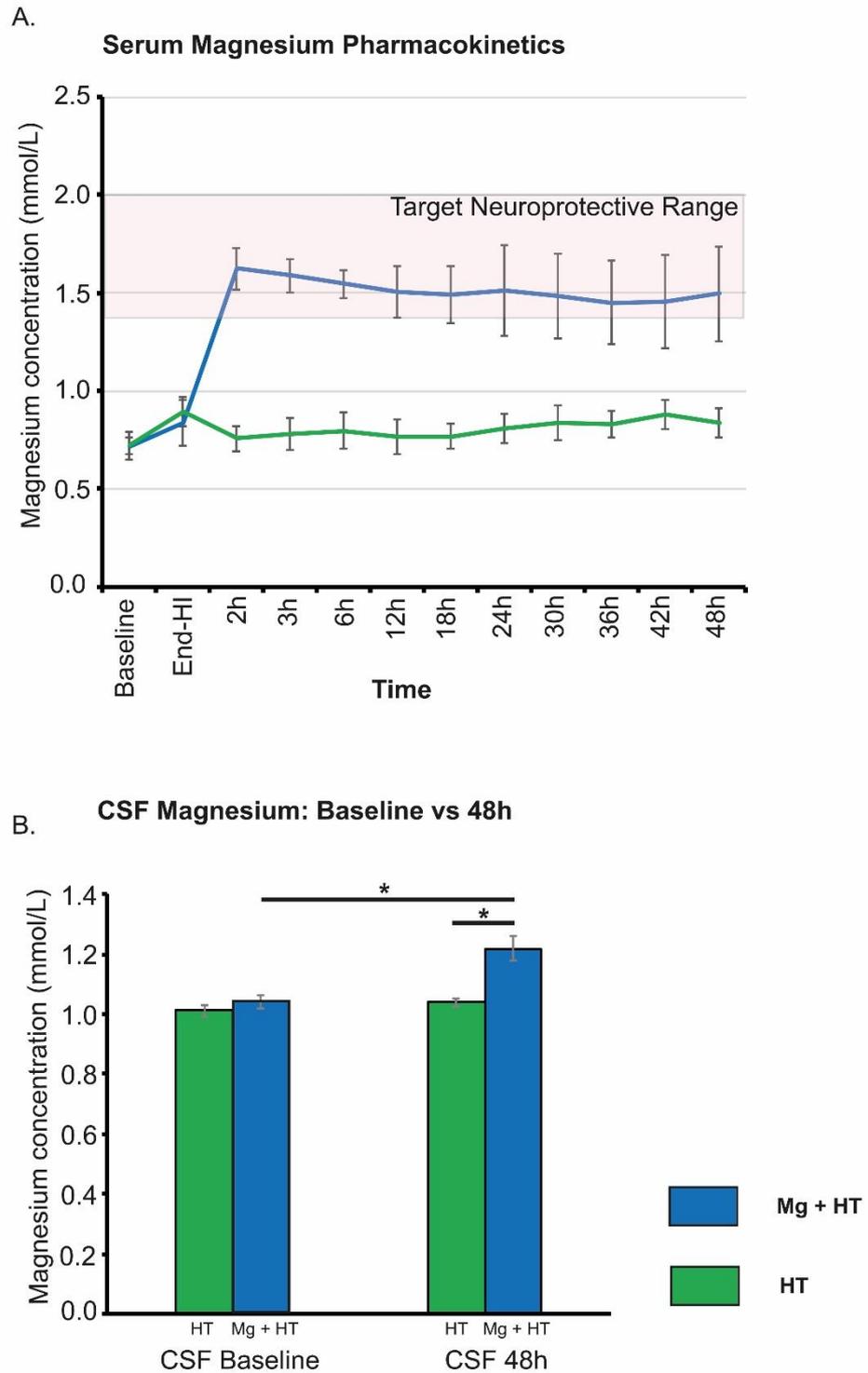


Figure 14. Pharmacokinetics following MgSO₄ bolus and infusion in serum (A) and CSF (B), (error bars represent SEM, *p<0.05).

*4.2.9 Results*

*$^1$H and $^{31}$P magnetic resonance spectroscopy*

$^1$H and $^{31}$P MRS was performed in 14 out of 15 piglets at 24 and 48 hours. There was no significant difference in MRS biomarkers of cerebral injury (white matter lac/NAA, thalamic lac/NAA, PCr/NTP, NTP/epp and PCr/Pi) between animals receiving MgSO$_4$ with HT and HT (Figure 15). Exclusion of animals with mild HI did not alter these findings (Appendix B2 / B3)**.**

**A.**

## Lac/NAA (White Matter)



**B.**

## Lac/NAA (Thalamus)



**C.**

## PCr/NTP



**D.**

## NTP/epp



**E.**

## PCr/P$_i$
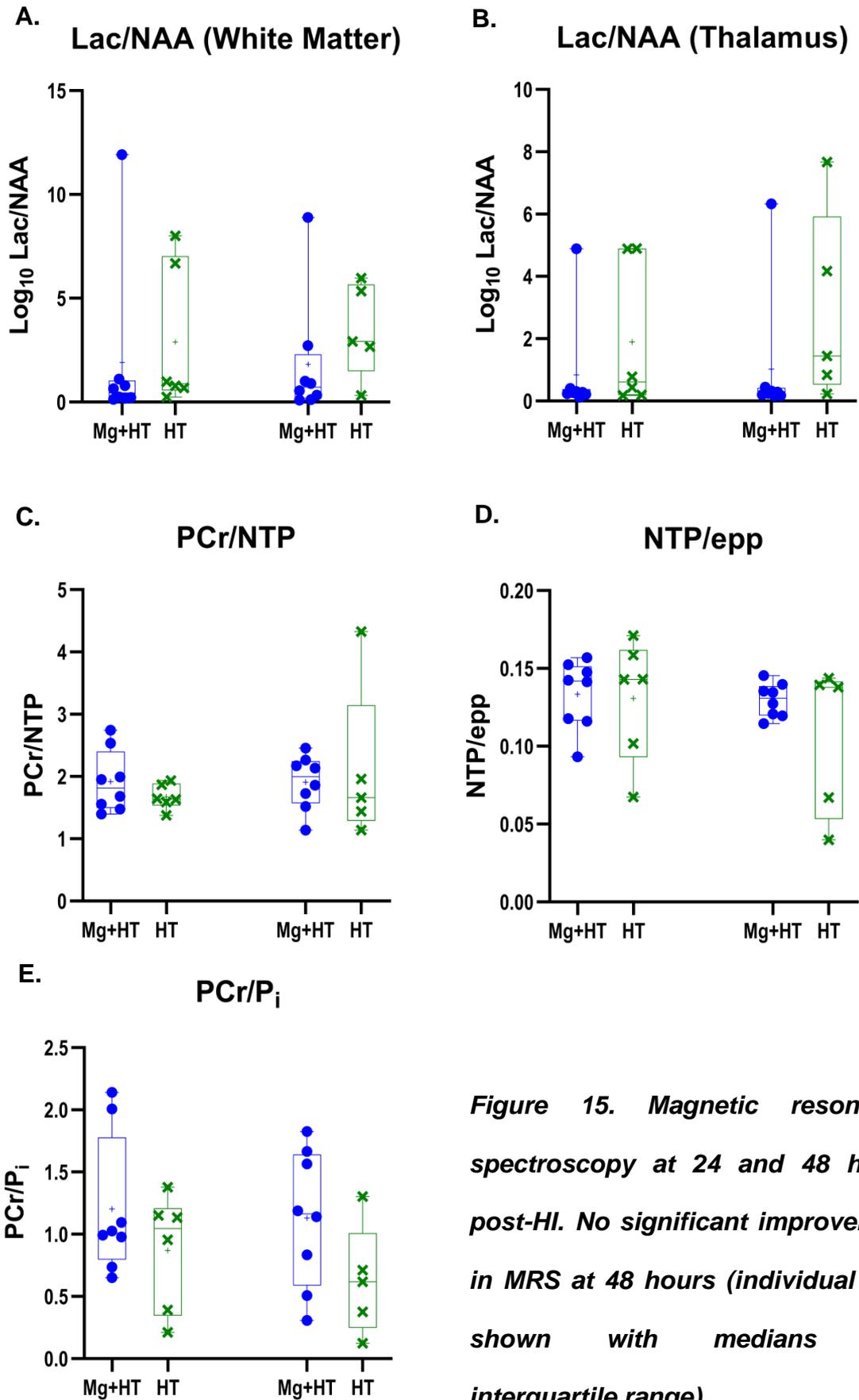


*Figure 15. Magnetic resonance spectroscopy at 24 and 48 hours post-HI. No significant improvement in MRS at 48 hours (individual data shown with medians and interquartile range).*

*Amplitude-integrated EEG*

Baseline aEEG was normal (score 4) in all animals prior to HI. Raw EEG activity typically became isoelectric (< 5µv) within 3 minutes of initiating the insult. Liberalising oxygen during insult to avoid severe hypotension did not result in EEG recovery. Two piglets (one in each group) demonstrated aEEG recovery to near normal (score 3) within 1 hour of HI, indicating a mild HI insult.

There was no significant difference between aEEG scores between Mg + HT and HT animals (p=0.09). Post-hoc analysis of moderate to severe HI (excluding animals with EEG recovery within 1 hour) however, demonstrated a statistically significant improvement in aEEG scores after 30 hours (Figure 16, Appendix B4 / B5)**.**

Two animals in each group developed seizures and were treated with phenobarbitone. There was no significant difference in number of hourly time epochs of seizures between Mg + HT animals and HT alone (10 hours vs 11 hours, p= 0.88).

**A.**



**aEEG Scoring over 48h (all piglets)**
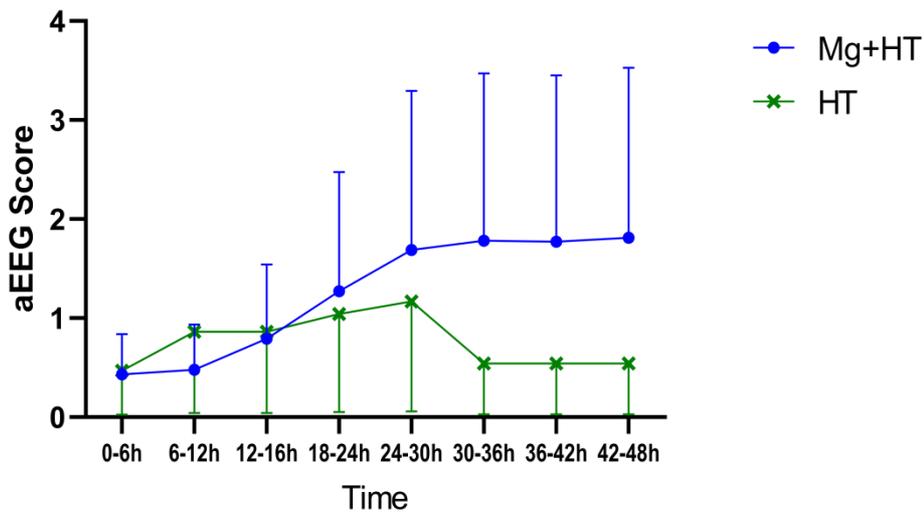
**B.**



**aEEG Scoring over 48h (moderate - severe HI)**
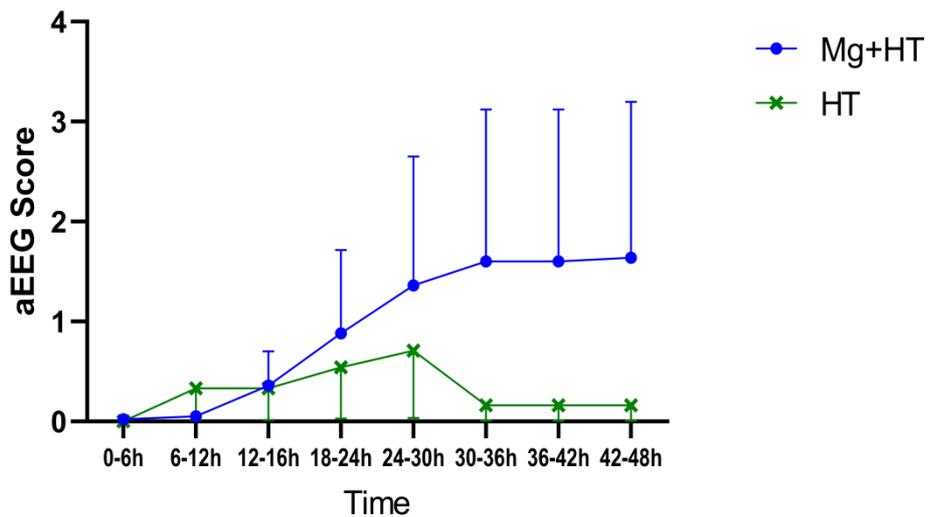
*Figure 16. Amplitude-integrated EEG scoring in Mg + HT compared to HT in all animals (A) and after exclusion of 2 piglets with mild injury (B). Error bars represent 95% CI (\*p<0.05).*

*Histology*

Immunohistochemical staining was undertaken in all 15 piglets and quantitative analysis performed between treatment groups. Analysis was performed between individual brain regions and overall (all regions).

TUNEL

Cell death was quantified using TUNEL staining; we observed a significant reduction in overall TUNEL positive cells in animals receiving Mg + HT compared to HT (21.1 vs 47.1 $\log_{10}$ count / $mm^2$, p = 0.014) (Figure 17). No significant improvement was seen in the periventricular white matter, cingulate cortex, sensorimotor cortex, hippocampus, internal capsule, caudate, putamen and thalamus. A post-hoc analysis excluding the two animals with mild HI did not alter these findings (Appendix B6 / B7).

Cleaved Caspase 3

Apoptosis was quantified by counting the number of CC3-positive cells. There was no significant difference in overall number of CC3-positive cells between animals in Mg + HT and HT groups (0.7 vs 0.5, p=0.29). Regional analysis demonstrated a significant increase in CC3-positive cells in the periventricular white matter in the Mg + HT group compared to HT alone (1.4 vs 0.6 $\log_{10}$ count/$mm^2$) (Figure 18A-D).

Iba1

Iba1 staining identifies microglia, which are ramified in their resting state and become amoeboid when activated. We observed a trend towards increased ramification (less activation) in Iba1 positive cells in animals treated with Mg + HT compared to HT (1.6 vs 1.1, p = 0.086). We did not identify any significant differences on analysis of individual brain regions (Figure 18E-H).

GFAP

Glial cells were stained using GFAP and quantified by measuring luminosity. There was no significant difference in overall GFAP optical luminosity between animals treated with Mg + HT compared to HT (64.7 vs 57.1, p = 0.11). Regional analysis demonstrated significantly higher luminosity in the cingulate cortex (59.2 vs 42.5, p=0.008) and caudate (49.7 vs 35.4, p=0.022) in Mg + HT animals compared to HT (Figure 18I-L).

OLIG2

Oligodendrocytes were stained and quantified by OLIG2 staining. There was a significant increase in overall OLIG2-positive cells in the Mg + HT animals compared to HT (2.44 vs 2.05 $\log_{10}$ count / mm$^2$, p = 0.002). Regional analysis demonstrated a significant increase in OLIG2-positive cells in the hippocampus (2.49 vs 1.67 $\log_{10}$ count / mm$^2$, p = 0.024) and thalamus (2.56 vs 1.6 $\log_{10}$ count / mm$^2$, p = 0.004) in the Mg + HT group compared to HT (Figure 18M-P).

**Overall TUNEL Count**
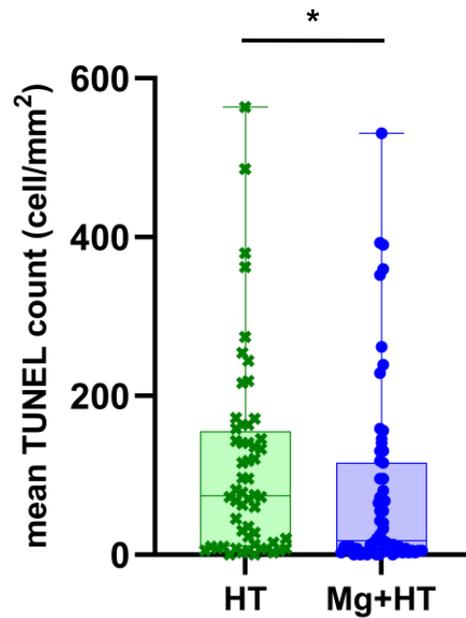


**Regional TUNEL Count**
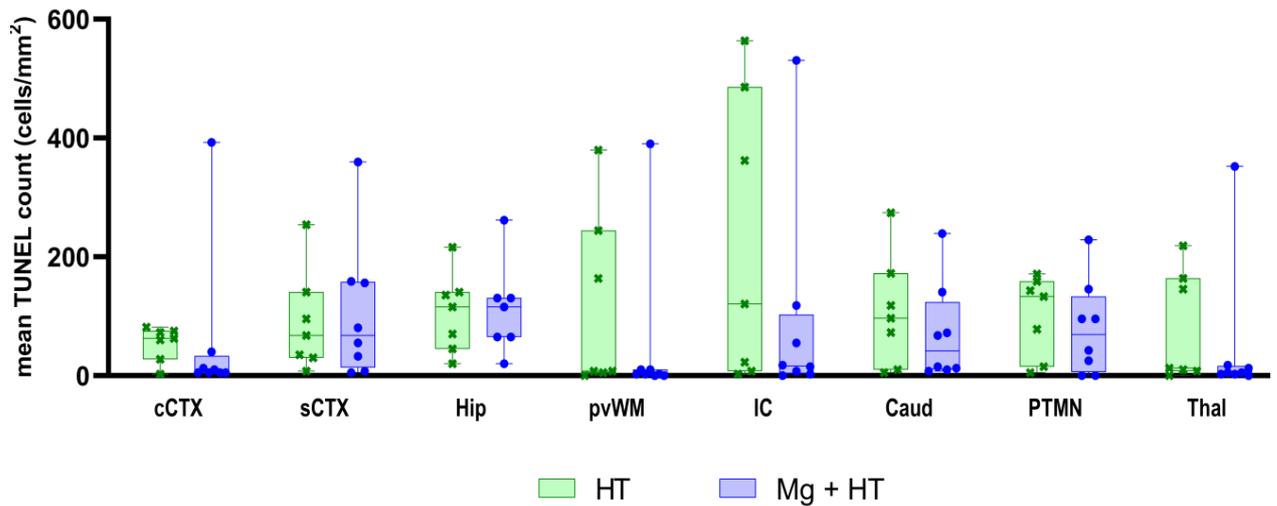


*Figure 17. Scatter plots with median and interquartile range for overall and regional brain cell death (TUNEL positive cell count). Significant reduction in overall cell death (p=0.014), particularly in periventricular white matter (p=0.094) in Mg + HT compared to HT (*p<0.05).*
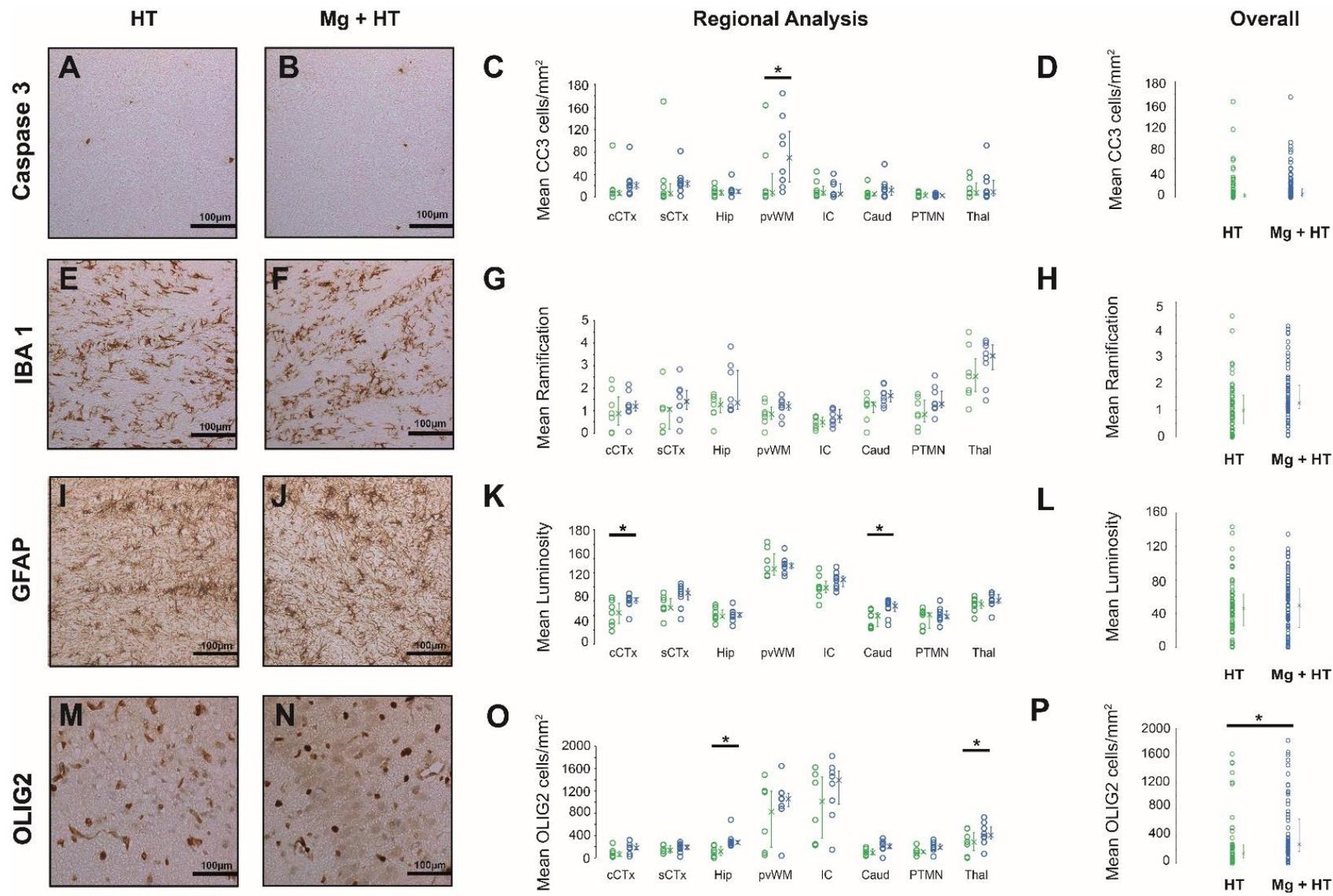
Figure 18. Scatter plots with median and interquartile range for overall and regional histology (cleaved caspase 3 (A-D), Iba1 ramification index (E-H), GFAP (I-L) and OLIG2 (M-P)).

cTX = cingulate cortex, sCTX = sensorimotor cortex, Hip = Hippocampus, pvWM = periventicular white matter, IC = internal capsule, Caud = caudate, PTMN = putamen, Thal = thalamus
(x = median; error bars represent interquartile range)

*p < 0.05

Mg + HT    HT

### 4.3 Discussion

$MgSO_4$ administered as a bolus and infusion is safe and provides a stable, suprasystemic serum magnesium concentration over 48 hours. We observed a significant reduction in cell death in animals treated with combined $MgSO_4$ and hypothermia. In animals with moderate to severe brain injury, there was a significant improvement in aEEG scores in the magnesium group. There was also an increase in surviving oligodendroglia in the hippocampus and thalamus in magnesium treated animals. There was however no significant benefit observed on MRS biomarkers at 24 or 48 hours after HI.

Hypotension is a common side effect of $MgSO_4$ and is dose dependent; repeated bolus doses of $MgSO_4$ increases the risk of hypotension [176], particularly at peak concentrations. This is a significant concern in encephalopathic infants who may have significant cardiovascular compromise. In a clinical study of hypothermia and $MgSO_4$, Rahman and colleagues reported no overall increase in inotrope requirements though they did not report cardiovascular parameters immediately following drug administration when serum concentrations would be highest [209]. Reassuringly, our findings confirm that raising serum magnesium to twice baseline levels is safe with no increase in inotrope requirements during hypothermia. The rise in CSF magnesium was modest (16%) compared to the increase observed in serum. Magnesium is actively transported into CSF via ATP-dependent ion exchangers and cation channels [224]. Saturation of these ion channels may limit the rate of magnesium transfer across the blood-brain barrier.

We observed a significant reduction in overall cell death in animals treated with Mg + HT compared to HT alone, particularly in the periventricular white matter. Magnesium is thought to be anti-excitotoxic and therefore may show greatest benefit in myelin-producing oligodendrocytes which are particularly sensitive to this mechanism of injury [225]. This is consistent with the observed increase in surviving oligodendria (OLIG2-positive cells) in animals receiving Mg + HT.

Prolonged infusions of $MgSO_4$ have previously been shown to reduce the number of mature oligodendrocytes in the intragyral and periventricular white matter in a preterm fetal sheep model [182]. We observed an increase in surviving oligodendria in magnesium exposed animals, though our data does not differentiate between immature and mature oligodendrocytes.

Of note, we observed an increase in CC3-postive cells in the periventricular white matter in animals receiving Mg + HT. This was a surprising finding given the trend towards improvement in TUNEL-positive cells observed in the same region. Caspases are crucial mediators of apoptosis and CC3 represents the principal protease in mammals. This discrepancy between TUNEL and CC3 may be due to the progressive nature of cell death following injury. Animals were sacrificed at 48 hours and therefore the full extent of cell death may not yet have fully evolved. Cell death may also be driven by caspase-independent pathways, as observed in models of ischemic stroke [226]. Mechanisms of apoptosis exhibit sexual dimorphism with male animals favouring caspase-independent mechanisms of cell death [227]. All piglets in this study were

male, which may explain why we have previously observed a good correlation of TUNEL-positive cells and weak correlation of CC3-positive cells with our primary translational MRS biomarker, Lac/NAA. This supports the use of TUNEL as the most appropriate outcome measure in this experimental model [228].

In a post-hoc sensitivity analysis excluding animals with mild HI, we observed a significant improvement in aEEG recovery in Mg + HT treated animals. Pattern classifications of aEEG provide a useful prognostic tool in NE and recovery of electrical activity has been associated with a good clinical outcome [229], though may be delayed in infants undergoing HT [109]. The recovery of aEEG in Mg + HT animals suggests additional efficacy was provided by combination therapy. $MgSO_4$ exposure however did not appear to reduce seizure burden, contrary to data from fetal sheep [230]. Although this may have been because isoflurane anaesthesia had masked the anti-epileptogenic effects of magnesium.

The primary outcome in this study was cerebral Lac/NAA, a robust prognostic MRS biomarker used in infants with NE [231,232] and surrogate measure of long-term outcomes in neonatal neuroprotection studies [153,233]. We did not observe improvement in lac/NAA in the thalamus or white matter. Together, these results suggest that the additional neuroprotective benefit provided by magnesium is incremental, too small to be identified using MRS biomarkers and therefore unlikely to provide substantial long-term benefit.

Magnesium is thought to be anti-inflammatory and has been shown to attenuate LPS-induced cytokine production *in vitro* [220] and *in vivo* [170]. In our study, there was no significant difference in overall microglial activation (Iba1) or astrogliosis (GFAP luminosity) and in fact, we observed an increase in astrogliosis in the cingulate cortex and caudate in the Mg + HT group. This suggests that magnesium does not alter immunological activation following HI. The effects of magnesium therapy on inflammatory cytokine gene expression is described in Chapter 5.

One of the main strengths of this study was the use of a large animal model of NE with a standardised HI, known timing of injury and clinically translatable outcome measures. The absence of observed benefit on MRS may in part be due to the small number of animals used, minimised in keeping with national guidance to reduce animal usage.

A limitation in this study was the reduction in duration of hypothermia to 12 hours, compared to 24 hours used in previous piglet studies [153]. We aimed to model a situation of incomplete hypothermic neuroprotection to better examine the impact of $MgSO_4$ on the evolution of neuronal injury, however additional hypothermia exposure may abolish the small neuroprotective benefit provided by magnesium. Furthermore, the exclusive use of male subjects in this study limits the generalisability of results due to gender-specific differences in responses to hypoxia and apoptotic pathways.

In conclusion, we have demonstrated baseline serum magnesium levels can be doubled safely and maintained over 48 hours using a $MgSO_4$ bolus and infusion. Combined Mg + HT therapy significantly reduced overall neuronal cell death, particularly in white matter and increased survival of oligodendrocytes. This neuroprotection is however incremental and given the absence of improvement seen on MRS, it is unlikely to translate to long-term benefit. The evolution of injury in NE is complex with several different mechanisms of injury. The key to improving outcomes may therefore lie in a cocktail of neuroprotective agents that work together to reduce injury and promote regeneration following perinatal asphyxia.

# Chapter 5

**Part (II): The effects of MgSO$_4$ on inflammatory gene**

**expression in hypothermia treated piglets with NE**

## 5.1 Methods

### 5.1.1 Method of HI

Hypoxia with ischaemia via transient carotid artery occlusion.

### 5.1.2 Study groups and protocol

The study protocol and timeline are detailed in Section 4.2.3. In this study, the gene expression (mRNA) of inflammatory cytokines and brain specific biomarkers were compared in animals exposed to $MgSO_4$ and hypothermia (Mg + HT) to those receiving hypothermia alone (HT). The panel of cytokines and brain biomarkers assessed are listed in Table 11.

| Target Gene | Function |
| --- | --- |
| Interleukin (IL) 1β | Pro-inflammatory cytokine. |
| Tumour necrosis factor α | Pro-inflammatory cytokine. |
| Interleukin (IL) 6 | Pro- and anti-inflammatory cytokine. |
| Interleukin (IL) 10 | Anti-inflammatory cytokine. |
| Chemokine ligand 2 (CCL2) | Gene script for monocyte chemoattractant protein 1 (MCP1), a chemokine that recruits monocytes, T cells and dendritic cells to sites of injury or infection. |
| Inducible Nitric Oxide Synthase (iNOS) | Induced in macrophages and microglia in response to inflammatory stimuli. |
| Glial fibrillary acidic protein (GFAP) | Cytoskeletal intermediate filament protein present in astrocytes. |
| S100B | Calcium binding protein and present in high concentrations in glial cells. |
| MAPT (Tau) | Gene for tau, a microtubule associated protein present in neurons of the central nervous system. |
| ENO2 (Enolase 2) | Cytosolic enzyme found in mature neurons. Marker of neuronal cell death. |

***Table 11. Cytokine and brain biomarker target genes (I): Mg + HT vs HT***

*5.1.3 Blood collection*

Blood samples were taken for gene expression at baseline and 2, 3, 6, 12, 24 and 48 hours post-HI. Whole blood (0.5 mL) was first mixed with acid-phenol, a denaturation solution which stabilises the RNA and inactivates RNAses and then stored at -80°C in cryovials for later RNA extraction.

*5.1.4 Isolating mRNA from whole blood*

*mRNA extraction*

Total RNA was extracted using the mirVANA miRNA Isolation Kit (Thermo Fisher Scientific, UK). Following pre-treatment with acid-phenol, blood samples were subjected to an acid-phenol: chloroform extraction which removed most other cellular components, including DNA. The RNA was then purified by a sequence of ethanol washes through a glass-fibre filter. RNA immobilised within this filter was then eluted with a nuclease-free, low ionic-strength solution.

*Measuring RNA concentration*

RNA quantification was performed using the Qubit RNA High Sensitivity (HS) Assay Kit (Thermo Fisher Scientific, UK). This assay involved binding a fluorescent dye to RNA targets and quantifying the concentration of RNA (ng/mL) using a pre-calibrated fluorometer.

*Reverse transcription*

RNA was converted to complementary DNA (cDNA) using a high capacity reverse transcription kit (Superscript$^{TM}$ VILO$^{TM}$, Thermo Fisher Scientific, UK). The kit included reverse transcriptase, primers, nucleotide triphosphate mix, buffers and a RNAse inhibitor. The reaction was performed in an automated thermal cycler; 10 minutes at 25$^o$C, 120 minutes at 37$^o$C and 85$^o$C for a final 5 minutes.

Two internal control samples were included: a sample without adding reverse transcriptase (minus RT control) in order to identify any genomic DNA contamination; and a reaction mix without sample (non-template control) to detect potential cross contamination between samples.

*mRNA amplification and analysis*

Real-time quantitative polymerase chain reaction (qRT-PCR) was used to amplify the cDNA sequence of interest using porcine-specific Taqman probes for IL1B (*IL1B*), IL6 (*IL6*), IL10 (*IL10*), tumour necrosis factor α (*TNFA*), chemokine CCL2/MCP2 (*CCL2*), inducible nitric oxide (iNOS), glial fibrillary acidic protein (*GFAP*), S100B (*S100B*), microtubule-associated protein tau (*MAPT*) and enolase 2 (*ENO2*). Two endogenous references genes ('house-keeping genes') were also included to allow standardisation; glyceraldehyde-3-phosphate dehydrogenase (*GAPDH)* and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ)* [153,234]. Target gene descriptions and assay ID are detailed in Appendix C1.

Customised 96-well plates (Thermo Fisher Scientific, UK) were pre-loaded with the supplied master mix (DNA polymerase, nucleotide triphosphates, buffers) and appropriate primers. Each target gene for all sample time points was analysed in triplicate. Relative fold-changes from baseline for each target gene was calculated using the ΔΔCT method [235].

*5.1.5 Calculating fold changes in gene expression*

When amplifying a product using PCR, the number of cycles taken to detect a threshold signal is known as the cycle quantification value (Cq value). In order to ensure that variations in Cq values between different experimental conditions are genuine, data are normalised to endogenous reference genes (GAPDH and YWHAZ). The ΔΔCT method compares the Cq values of the target gene to the Cq values of endogenous reference genes. This provides a method to calculate the relative changes in gene expression at specific time points from baseline (see below):

Relative Quantification (fold-change) = $2^{-\Delta\Delta CT}$

$\Delta\Delta CT = \Delta CT_{\text{time point } x} - \Delta CT_{\text{baseline}}$

$\Delta CT_{\text{baseline}} = Cq_{\text{Target gene at baseline}} - Cq_{\text{ETC at baseline}}$

$\Delta CT_{\text{time point } x} = Cq_{\text{Target gene at } x \text{ time point}} - Cq_{\text{ETC at } x \text{ time point}}$

Cq = cycle quantification value for specific genes

ETC = endogenous control gene

*5.1.6 Statistical analysis*

Average baseline Cq values for each gene was calculated using baseline samples from all study groups. The Cq value of the gene of interest in individual study groups was then compared to this common baseline Cq value. Raw data was $\log_{10}$ transformed to account for skewness and a value of 0.05 was added to all results to allow analysis of zero values. Results were fitted to an ANOVA model; fitting terms included treatment, time (as a factor) and treatment by time interaction. Comparisons in fold-changes were made between study groups at each individual time point. No adjustments were made for repeated measures.

**5.2 Results**

Baseline characteristics, physiological data and severity of HI are described in Section 4.2.6. In both study groups, *IL1B*, *TNFA*, *IL6* and *IL10* mRNA levels peaked within 3 hours and *CCL2* mRNA within 6 hours post-insult (Figure 19, Appendix C2). There were no statistically significant differences in inflammatory cytokine gene expression between Mg + HT compared to HT animals at any time points. One animal in the HT group had a large increase in *CCL2* and *TNFA* mRNA at 48 hours, skewing the data at this time point.
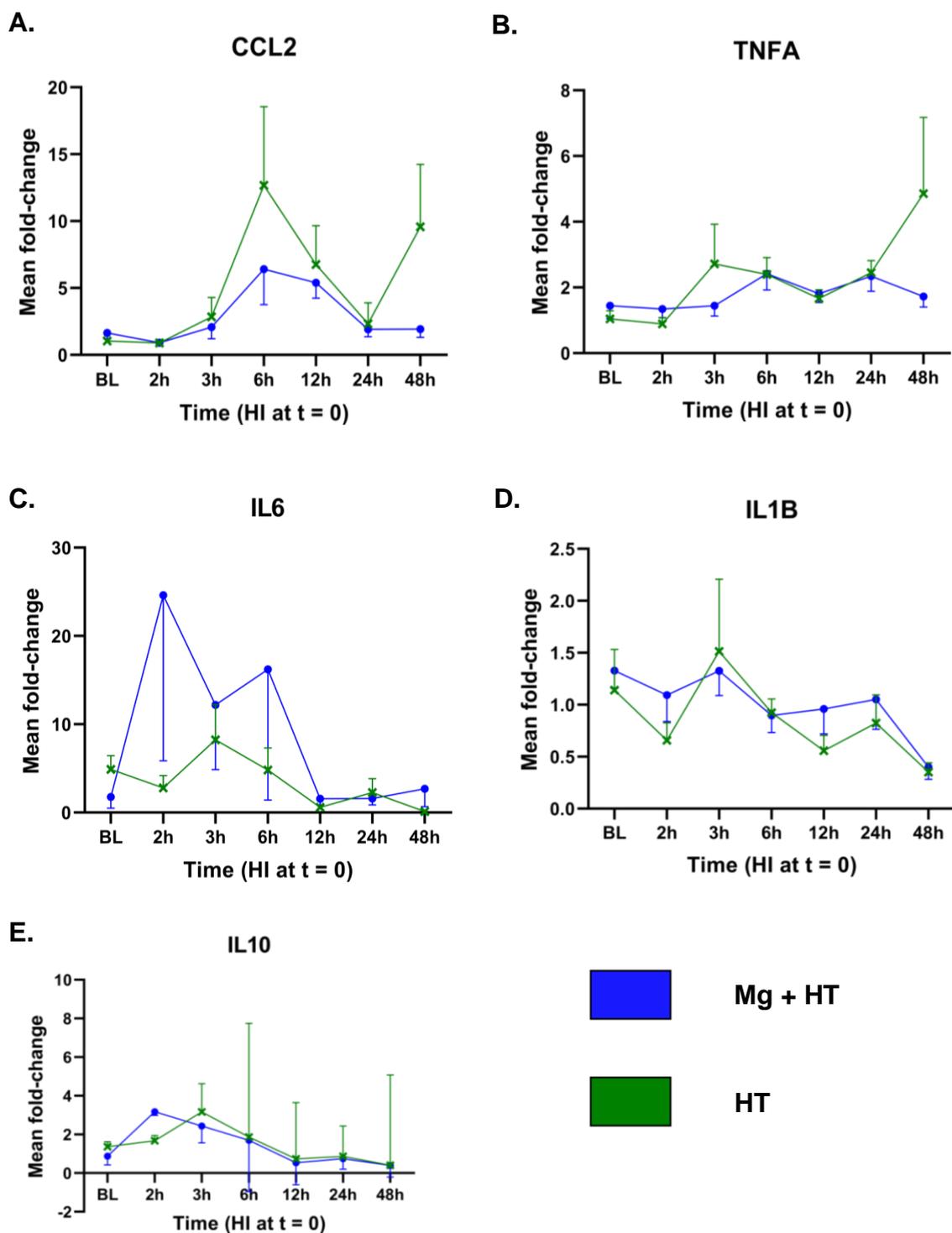
*Figure 19. Serum mRNA transcript levels of pro-inflammatory chemokine (A), cytokines (B-D) and anti-inflammatory cytokine (E) over 48 hours. There were no significant differences in cytokine or chemokine mRNA transcript levels between study groups (error bars represent SEM).*

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*ENO2*, *iNOS* and *MAPT* mRNA were increased in both Mg + HT and HT animals within 3 hours of insult and decreased to baseline levels within 12 hours. Both *S100B* and *ENO2* mRNA levels increased at 24 and 48 hours, indicating these may be late biomarkers of injury (Figure 20). *GFAP* mRNA levels were below the threshold of detection in most sample time points and therefore insufficient for analysis.
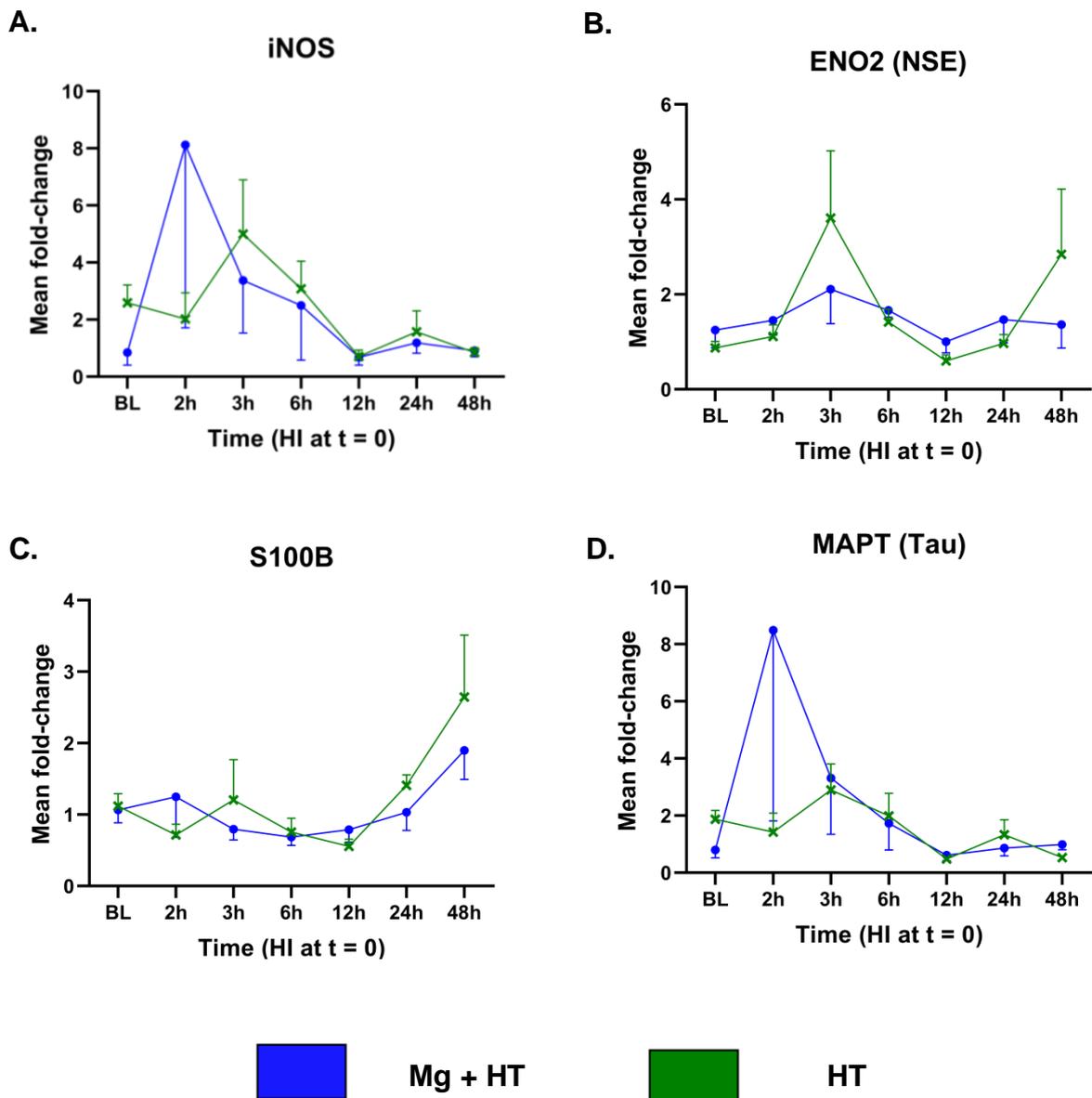


*Figure 20. Serum mRNA transcript levels of iNOS (A), ENO2 (B), S100B (C) and MAPT (D) over 48 hours (error bars represent SEM).*

## 5.3 Discussion

This experiment explored the immunomodulatory effects of $MgSO_4$ in combination with HT over 48 hours after HI. Inflammatory cytokine expression increased rapidly after insult; *IL1B, TNFA, IL6* and *IL10* mRNA levels peaked at 3 hours and *CCL2* mRNA at 6 hours in both study groups. $MgSO_4$ exposure however did not alter the magnitude or timing of serum cytokine gene expression.

Inflammation is a well-established mechanism of injury in preterm brain injury and it has been suggested that the success of $MgSO_4$ in this population may be attributed to its anti-inflammatory properties. There is limited data exploring whether magnesium has direct anti-inflammation properties. In vitro analysis of maternal monocytes exposed to magnesium, demonstrated decreased LPS-induced IkBα mRNA, reduced NF-kB phosphorylation and decreased nuclear NF-kB levels [220]. In an *ex vivo* placental model, LPS-induced NF-kB and IL6 expression was significantly reduced when given in combination with $MgSO_4$ [236]. Attenuation of cytokine expression however does not necessarily translate to reduction in inflammation-mediated injury. In a fetal rodent model, magnesium improved the learning ability of offspring following maternal LPS exposure [171]. This study however was limited to males only and did not assess histological outcomes. Experimental data supporting magnesium as modulator of inflammation has not borne out in clinical trials. In a secondary analysis of the BEAM trial focussing on women with clinical signs

of chorioamnionitis, magnesium did not significantly reduce rates of still birth, death or moderate-severe cerebral palsy [174].

In our study, magnesium exposure did not alter the gene expression of inflammatory cytokines. One possibility is that the immunomodulatory effects hypothermia masked the anti-inflammatory properties of magnesium. Alternatively, magnesium may not be anti-inflammatory and the reduction in neuronal cell death and improved aEEG scores in moderate to severe brain injury were mediated via NMDA receptor activity or perhaps, indirectly through vasodilation.

NOS catalyses the production of nitric oxide (NO), a biological intercellular messenger with a wide range of physiological and pathological effects. Post-asphyxia, nitric oxide itself or in combination with superoxide free radicals is neurotoxic; disrupting protein structures, damaging mitochondrial function and inducing apoptosis [237]. Three isoforms of this enzyme exist, neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). Inducible NOS is primarily produced in macrophages, microglia, neutrophils and neurons. $MgSO_4$ is known to alter NOS expression in LPS stimulated pregnant rodents [238] and LPS stimulated placentas *ex vivo* [236]; suggesting that magnesium reduces inflammation by inhibiting NOS. In this study, magnesium exposure did not appear to alter iNOS mRNA expression with transcript levels increasing in both groups within 3 hours post-HI.

This study demonstrates the feasibility of detecting mRNA transcripts of cytokines and brain biomarkers in the serum in the first few hours following HI. The presence of brain biomarker mRNA in the serum suggest disruption to the blood brain barrier, permitting the release of these biomarkers into the systemic circulation. Interestingly, *GFAP* mRNA was undetectable in several samples in both study groups, suggesting that transcript levels were below the threshold of detection using PCR.

One of the main strengths of this study was the precise timing and delivery of a standardised HI in this model, allowing the accurate mapping of biomarker expression following injury. The early rise in *MAPT*, *iNOS* and *ENO2* mRNA and late rise in *S100B* mRNA following HI may be useful in establishing the timing of cerebral injury in the hours after birth. The presence of MAPT, iNOS and ENO2 mRNA is indicative of a recent injury, whereas rising *S100B* mRNA soon after birth would suggest that injury preceded delivery by several hours. This study highlights the potential for gene expression studies to interrogate several serum biomarkers simultaneously and support clinicians in estimating the timing of injury in infants with NE.

One of the limitations of this study is the small numbers of animals involved and therefore fold-changes in gene expression were sometimes skewed by individual animals with exceptionally raised mRNA transcript levels. Furthermore, mRNA expression may not correlate with protein levels due to the complex post-transcriptional modifications that follow hypoxia and inflammation [239]. It is therefore difficult to extrapolate pathophysiological

mechanisms using serum gene expression data. Furthermore, in the absence of sham gene expression data, we are unable to determine the impact of surgery on the cytokine mRNA profile as well as understand the influence of hypothermia on the inflammatory response.

In conclusion, $MgSO_4$ exposure did not alter inflammatory cytokine mRNA expression, iNOS or brain biomarkers of injury in hypothermic piglets following HI. Further pre-clinical study of carefully selected serum and brain cytokine proteins may provide further insight into the effects of magnesium exposure on the inflammatory cascade.

In order to improve upon hypothermic neuroprotection, attention must be directed both at adjunctive agents as well as refining our understanding of the disease itself. Though inflammation is known to exacerbate perinatal brain injury, the clinical phenotype of infants exposed to ascending intra-uterine infection prior to perinatal asphyxia may be identical to those with a pure hypoxic ischaemic injury. Currently, the same treatment strategies are applied to all infants who meet the criteria for therapeutic hypothermia despite recent evidence to suggest reduced efficacy in the presence of infection [240]. Further defining the underlying pathological processes in this group of infants is the first step in developing precision medicine and patient-specific therapy. In the following chapter, we use similar advanced biological techniques to differentiate hypoxia, inflammation and combined inflammation with hypoxia brain injury in a novel piglet model of inflammation-sensitised NE.

# Chapter 6

## Part (III): Differentiating Biomarkers of Inflammation and Hypoxia Brain Injury

## 6.1 Methods

This study has now been published (see publication- Appendix A6).

### 6.1.1 Method of HI

This experiment coincided with a change in insult paradigm, moving to a global hypoxia insult with hypotension-induced ischaemia (i.e. no carotid artery occlusion). This was done to reduce the potential risk of accidental cerebral ischaemia during placement of carotid occluders.

### 6.1.2 Study groups

Following surgery and baseline observation, piglets were randomised by a computer generated sequence to receive either (i) LPS, n = 5 (LPS 2µg/kg bolus and 1µg/kg infusion for 52 hours); (ii) Hypoxia, n = 6 (saline with hypoxia); or (iii) LPS + Hypoxia, n = 5 (LPS 2µg/kg bolus and 1µg/kg infusion commencing 4 hours prior to hypoxia) (Figure 21)**.**
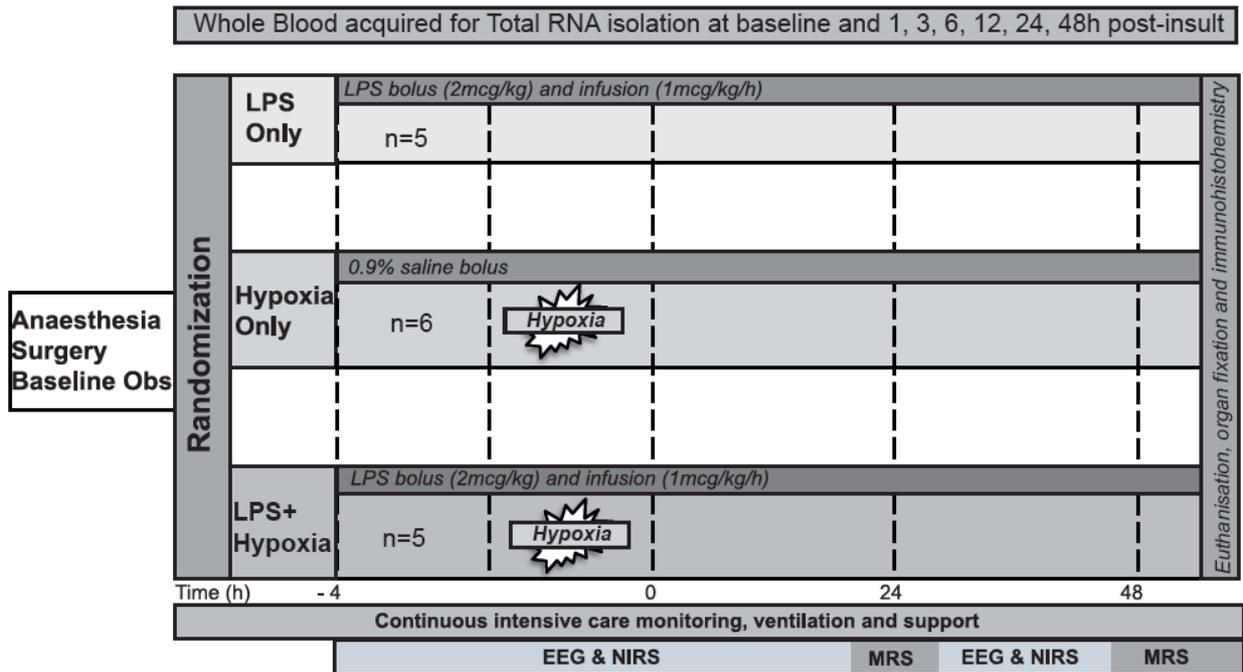
**Figure 21. Study timeline (II): 16 piglets randomised following a hypoxia insult receive either (i) LPS (n=5), (ii) Hypoxia (n=6) or (iii) LPS + Hypoxia (n=5). Animals euthanised at 48 hours.**

*6.1.3 Biomarker targets*

Based on a review of the literature and in collaboration with developmental neurobiologists at the Centre for Developing Brain (Kings College London), we identified several inflammatory cytokines and brain-specific biomarkers that have previously been used as markers of cerebral injury or prognostic indicators in infants with NE (Table 12). These were then assessed to determine if they can differentiate between inflammation and hypoxia mediated injury.

| Target Gene | Function |
|---|---|
| Interleukin (IL) 1α, 6, 8 | Pro-inflammatory cytokine. |
| Tumour necrosis factor (TNF) α | Pro-inflammatory cytokine. |
| Chemokine ligand 2 (CCL2) | Gene script for Monocyte Chemoattractant protein 1 (MCP1), a chemokine that recruits monocytes, T cells and dendritic cells to sites of injury or infection. |
| Interleukin (IL) 10 | Anti-inflammatory cytokine. |
| Brain derived Neurotrophic factor (BDNF) | Supports neuronal survival and encourages growth and differentiation. |
| Glial fibrillary acidic protein (GFAP) | Cytoskeletal filament protein present in astrocytes. |
| Ubiquitin carboxyl-terminal hydrolase L1 (UCHL1) | Neuron specific enzyme that is used as a marker of apoptosis. |
| S100B | Calcium binding protein; present in glial cells. |
| Microtubule associated protein tau (MAPT) | Gene for tau, a microtubule associated protein present in neurons of the central nervous system. |
| Enolase 2 (ENO2; Neuron specific enolase) | Cytosolic enzyme found in mature neurons. Marker of neuronal cell death. |

***Table 12. Cytokine and brain biomarker target genes (II): LPS vs Hypoxia vs LPS + Hypoxia***

*6.1.4 Blood collection*

Blood samples were taken at baseline, 4 hours after the LPS bolus, immediately following hypoxia and then at 1, 3, 6, 12, 24 and 48 hours post-hypoxia. Whole blood (0.5 mL) was mixed with acid-phenol and stored at -80°C for later RNA extraction.

*6.1.5 mRNA isolation and analysis*

Total mRNA was extracted, quantified and converted to cDNA as described in Section 5.1.4.

Genes of interest were quantified by qRT-PCR using porcine-specific Taqman probes for IL1A (*IL1A*), IL6 (*IL6*), IL8 (*CXCL8*), IL10 (*IL10*), tumour necrosis factor α (*TNFA*), chemokine CCL2/MCP2 (*CCL2*), brain derived neurotrophic factor (*BDNF*), glial fibrillary acidic protein (*GFAP*), ubiquitin carboxyl-terminal hydrolase L1 (*UCHL1*), S100B (*S100B*), microtubule-associated protein tau (*MAPT*) and enolase 2 (*ENO2*).

Three endogenous references genes were included for standardisation; glyceraldehyde-3-phosphate dehydrogenase (*GAPDH)*, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (*YWHAZ)* and peptidylprolyl isomerase A (*PPIA)* [153,234,241]. The Cq values of the endogenous control genes were not significantly different between LPS, Hypoxia and LPS + Hypoxia groups, indicating that these genes were unaffected by the different experimental conditions.

Each target gene for all sample time points was analysed in triplicate and relative fold-changes from baseline for each gene was calculated using the ΔΔCT method (see Section 5.1.5). Fold-changes at specific time-points were compared to a 'common' baseline group, created using gene data from all animals in the study (n=16). This approach allowed a larger sample size to represent pre-insult mRNA levels. This also provided a common denominator

to allow comparisons to be made between injury subtypes at specific time points.


### 6.1.6 miRNA microarray analysis

Total RNA samples were processed by UK Bioinformatics Ltd on miRNA 4.0 arrays as per manufacturer's instructions (Thermo Fisher Scientific, UK). In brief, samples were labelled using the FlashTag™ Biotin HSR Labelling Kit (Thermo Fisher Scientific, UK) and hybridised to GeneChip miRNA 4.0 microarrays (Affymetrix, UK), designed to interrogate all mature miRNA sequences in miRBase, Release 20. The arrays were loaded into a hybridisation oven (GeneChip Scanner 3000 7G system) and incubated at 48°C and 60 rpm for 16 - 18 hours.

Analysis was performed using the Affymetrix Expression Console™ software for data normalisation and quality control. The fluorescent signals generated by specific miRNA were compared to baseline values from all study groups. Differential miRNA expression analysis was performed using the Transcriptome Analysis Console (TAC v4.0) software. Results were filtered to highlight statistically significant fold changes. As this was an exploratory experiment, a False Discovery Rate (FDR) p-value < 0.1 was used to identify miRNA of interest. Human and porcine miRNA with identical sequences were removed. These miRNAs were evaluated for significance (p-value < 0.05) at specific time points between groups as well as fold-changes across time within group.

### 6.1.7 Statistical analysis

Physiological data was analysed using ANOVA (Tukey-Kramer multiple comparison correction). Pairwise comparisons were made using a T test or Mann Whitney U test for parametric and non-parametric data, respectively.

Analysis of mRNA and miRNA levels was performed as stated in Section 5.1.6. In brief, fold-changes in mRNA and miRNA levels at specific sample time points was compared to an averaged baseline value calculated from all study groups combined. Gene expression data (mRNA, miRNA) was $\log_{10}$ transformed for normalisation and analysed using an ANOVA model. Pairwise correlations (R2) was made between mean overall TUNEL score ($\log_{10}$ cells per $mm^2$) for each animal versus target mRNA and miRNA fold-changes at each time point. These results were assessed and plotted. No adjustments were made for repeated measures.

In the event of cardiac arrest prior to experiment completion, mRNA transcription and miRNA data from preceding time points were still included in the analysis. Fold changes in target mRNA and miRNA levels were correlated with regional and overall TUNEL counts, representing neuronal cell death across all brain regions (pairwise correlation).

### 6.1.8 Sample size calculation

Group sizes were estimated using data from previous piglet studies and 2 pilot LPS experiments. At least 5 animals in each group were needed to detect a difference of 45 TUNEL positive cells / $mm^2$, using a significance threshold of 5% and 80% power.

## 6.2 Results

### 6.2.1 Baseline characteristics and physiological parameters

Sixteen large white male piglets aged less than 36 hours were studied. All animals appeared healthy prior to experimentation with similar mean weight (1960g; range 1650 - 2100g) between study groups (p = 0.216). Animals were monitored for at least 1 hour following surgery and baseline physiological parameters (HR, MBP, Temp) were within acceptable limits (Table 13). Of note, animals in the hypoxia group had slightly alkalotic blood gases with lower pCO2 at baseline. All other differences in physiological parameters were within normal limits.

### 6.2.2 Hypoxia insult

Insult severity was similar between the Hypoxia and LPS + Hypoxia groups. There was no significant difference between the duration of hypoxia, hypotension (MABP < 27mmHg), isoelectric EEG and area under the curve (AUC) $FiO_2$ between groups (Table 14). There was a trend towards a shorter duration of EEG < 5µV during insult in the LPS + Hypoxia group compared to the Hypoxia group (p = 0.07). End of insult blood gases were similar between groups.

| | LPS | | Hypoxia | | LPS + Hypoxia | | |
|---|---|---|---|---|---|---|---|
| | *Mean* | *SD* | *Mean* | *SD* | *Mean* | *SD* | *p-value* |
| **Weight (grams)** | 2020 | 130 | 1967 | 137 | 2010 | 114 | |
| **Temperature (ºC)** | | | | | | | |
| Baseline | 38.7 | 0.94 | 38.3 | 0.78 | 38.4 | 0.63 | 0.70 |
| 4hLPS | 38.9 | 0.12 | 39.0 | 0.25 | 38.9 | 0.10 | 0.56 |
| End of insult (t=0) | 38.6 | 0.24 | 38.5 | 0.26 | 38.1 | 0.30 | **0.02*** |
| 12h | 38.4 | 0.16 | 39.0 | 0.29 | 38.8 | 0.27 | **0.004*** |
| 24h | 38.3 | 0.21 | 38.5 | 0.25 | 38.5 | 0.10 | 0.24 |
| 48h | 38.4 | 0.20 | 38.4 | 0.24 | 38.3 | 0.0 | 0.81 |
| **Heart rate (bpm)** | | | | | | | |
| Baseline | 179 | 16.9 | 170 | 9.7 | 176 | 18.7 | 0.60 |
| 4hLPS | 210 | 18.0 | 186 | 14.6 | 208 | 8.3 | **0.02*** |
| End of insult (t=0) | 218 | 19.7 | 209 | 9.3 | 222 | 14.3 | 0.40 |
| 12h | 208 | 19.0 | 217 | 15.6 | 224 | 12.2 | 0.34 |
| 24h | 207 | 22.1 | 201 | 31.9 | 210 | 16.5 | 0.86 |
| 48h | 175 | 27.6 | 166 | 45.7 | 175 | 11.4 | 0.90 |
| **MABP (mmHg)** | | | | | | | |
| Baseline | 50 | 4.1 | 46 | 5.7 | 55 | 3.5 | **0.02*** |
| 4hLPS | 50 | 6.1 | 43 | 4.3 | 54 | 3.1 | **0.006*** |
| End of insult (t=0) | 45 | 9.0 | 50 | 10.2 | 50 | 24.1 | 0.79 |
| 12h | 44 | 85 | 45 | 9.5 | 47 | 18.8 | 0.94 |
| 24h | 49 | 1.9 | 46 | 3.7 | 42 | 6.0 | 0.08 |
| 48h | 55 | 3.9 | 52 | 6.4 | 55 | 3.1 | 0.61 |
| **pH** | | | | | | | |
| Baseline | 7.38 | 0.08 | 7.55 | 0.09 | 7.43 | 0.05 | **0.01*** |
| 4hLPS | 7.40 | 0.08 | 7.49 | 0.08 | 7.42 | 0.05 | 0.14 |
| End of insult (t=0) | 7.46 | 0.10 | 7.10 | 0.13 | 7.09 | 0.05 | **0.006*** |
| 12h | 7.48 | 0.10 | 7.47 | 0.13 | 7.49 | 0.09 | 0.98 |
| 24h | 7.41 | 0.04 | 7.45 | 0.04 | 7.27 | 0.29 | 0.2 |
| 48h | 7.47 | 0.07 | 7.42 | 0.05 | 7.49 | 0.08 | 0.26 |
| **pCO2 (kPa)** | | | | | | | |
| Baseline | 6.8 | 1.6 | 4.4 | 0.7 | 6.2 | 1.1 | **0.01*** |
| 4hLPS | 6.3 | 1.1 | 5.4 | 1.0 | 5.6 | 0.7 | 0.28 |
| End of insult (t=0) | 5.9 | 1.6 | 6.0 | 1.0 | 5.6 | 1.2 | 0.85 |
| 12h | 5.3 | 1.1 | 6.2 | 2.6 | 4.9 | 0.6 | 0.51 |
| 24h | 6.2 | 0.4 | 5.1 | 1.3 | 4.7 | 0.3 | 0.07 |
| 48h | 5.0 | 0.7 | 5.5 | 0.9 | 4.5 | 0.2 | 0.21 |

| pO2 (kPa) | | | | | | | |
|---|---|---|---|---|---|---|---|
| Baseline | 13.6 | 3.6 | 10.7 | 1.1 | 15.1 | 4.9 | 0.14 |
| 4hLPS | 10.8 | 1.7 | 11.1 | 1.8 | 10.1 | 0.9 | 0.59 |
| End of insult (t=0) | 12.0 | 0.6 | 6.8 | 3.5 | 7.4 | 3.5 | 0.20 |
| 12h | 12.9 | 2.5 | 14.3 | 5.2 | 11.5 | 0.7 | 0.51 |
| 24h | 9.0 | 0.6 | 10.5 | 1.9 | 10.6 | 3.2 | 0.43 |
| 48h | 11.9 | 3.2 | 12.2 | 2.2 | 13.0 | 2.5 | 0.90 |
| **BE** | | | | | | | |
| Baseline | 5.2 | 2.2 | 6.2 | 2.9 | 6.2 | 1.9 | 0.76 |
| 4hLPS | 4 | 1.4 | 6.3 | 1.8 | 2.8 | 2.2 | **0.02*** |
| End of insult (t=0) | 6.5 | 0.7 | -15.3 | 5.9 | -17 | 2.9 | **0.001*** |
| 12h | 6.4 | 3.5 | 8.3 | 1.2 | 4.5 | 5.8 | 0.29 |
| 24h | 4.6 | 2.3 | 2.5 | 5.9 | -7.8 | 14.9 | 0.11 |
| 48h | 2.8 | 1.5 | 2.3 | 5.5 | 2.5 | 4.9 | 0.98 |
| **Lactate** | | | | | | | |
| Baseline | 4.0 | 1.4 | 5.6 | 1.2 | 3.4 | 1.4 | **0.045*** |
| 4hLPS | 4.4 | 0.7 | 4.2 | 1.1 | 5.4 | 1.6 | 0.25 |
| End of insult (t=0) | 4.1 | 0.8 | 16.0 | 2.9 | 15.9 | 0.8 | **0.0001*** |
| 12h | 3.0 | 1.1 | 2.6 | 0.7 | 5.2 | 3.5 | 0.13 |
| 24h | 1.6 | 0.3 | 4.3 | 3.9 | 9.3 | 8.0 | 0.09 |
| 48h | 0.9 | 0.3 | 1.5 | 0.8 | 1.1 | 0.3 | 0.27 |
| **Glucose** | | | | | | | |
| Baseline | 6.9 | 1.2 | 5.8 | 1.1 | 5.2 | 1.9 | 0.21 |
| 4hLPS | 7.0 | 1.6 | 8.6 | 1.4 | 6.5 | 2.2 | 0.26 |
| End of insult (t=0) | 6.7 | 1.3 | 10.7 | 2.9 | 10.5 | 3.8 | 0.33 |
| 12h | 5.8 | 0.9 | 7.4 | 1.6 | 6.6 | 1.3 | 0.18 |
| 24h | 6.1 | 0.4 | 9.4 | 5.6 | 9.2 | 3.7 | 0.39 |
| 48h | 5.5 | 0.3 | 6.3 | 1.7 | 5.4 | 0.8 | 0.54 |

*Table 13. Physiological data and blood gas analysis (II), (mean, SD)*

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|  | Hypoxia | | LPS + Hypoxia | | |
|---|---|---|---|---|---|
|  | *Mean* | *SD* | *Mean* | *SD* | *p-value* |
| Duration (min) | 32 | 10 | 24.4 | 4.5 | 0.14 |
| AUC FiO2 (%) | 468 | 177 | 357 | 67 | 0.17 |
| Duration of EEG<5uV (min) | 27.3 | 9.6 | 16.4 | 6.9 | 0.07 |
| Duration of MBP < 30 (min) | 15 | 8.2 | 8.8 | 4.8 | 0.16 |
| End of Insult Gas: | | | | | |
| pH | 7.10 | 0.13 | 7.09 | 0.03 | 0.92 |
| pCO2 | 6.0 | 1.0 | 5.6 | 1.2 | 0.56 |
| BE | -15.3 | 5.9 | -17 | 2.9 | 0.62 |
| Lactate | 16.0 | 2.9 | 15.9 | 0.8 | 0.93 |

*Table 14. Hypoxia ischaemia insult parameters (II), (mean, SD)*

*6.2.3 Survival and clinical illness severity*

As reported in Martinello et al. [94], mortality was significantly increased in animals receiving LPS + Hypoxia; three of the five LPS + Hypoxia piglets died compared with none in the other groups (p = 0.022). One piglet died within minutes of insult cessation. Two further piglets arrested at approximately 24 hours with preceding refractory hypotension. Two animals (one LPS, one Hypoxia piglet) were successfully resuscitated following mechanical airway obstruction / equipment failure and survived to experiment completion.

There was no significant difference in inotrope use or saline bolus requirement between groups (Table 15). The Hypoxia and LPS + Hypoxia groups required higher doses of dopamine, dobutamine, adrenaline and saline than non-hypoxia groups (p ≤ 0.046).

| | LPS | | Hypoxia | | LPS+Hypoxia | | |
|---|---|---|---|---|---|---|---|
| | *Mean* | *SD* | *Mean* | *SD* | *Mean* | *SD* | *p-value* |
| Dopamine (mcg/kg/min) | 3.6 | 2.1 | 8.9 | 8.3 | 13.4 | 6.0 | 0.15 |
| Dobutamine (mcg/kg/min) | 0 | 0 | 4.8 | 7.0 | 4.8 | 4.8 | 0.34 |
| Noradrenaline (ng/kg/min) | 0 | 0 | 34.9 | 56.4 | 18.6 | 19.0 | 0.41 |
| Adrenaline (ng/kg/min) | 0.9 | 1.9 | 147.9 | 229.8 | 169.6 | 151.2 | 0.31 |
| 10ml/kg Saline Bolus (n) | 0 | 0 | 0.5 | 0.7 | 0.4 | 0.4 | 0.12 |

***Table 15. Volume and inotrope requirements (II), (mean, SD)***

*6.2.4 Gene expression for cytokines and chemokines*

Samples for gene expression and miRNA were available for all animals in the LPS and Hypoxia groups. In the LPS + Hypoxia group, serum was available for 4/5 animals until 24 hours post-insult and 2/5 animals at 48 hours (Table 16).

| | | *Number of samples analysed at each time points* | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | **Total number of piglets** | **Baseline** | **4h after LPS bolus** | **1h** | **3h** | **6h** | **12h** | **24h** | **48h** |
| **LPS** | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| **HYPOXIA** | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| **LPS + HYPOXIA** | 5 | 4 | 4 | 4 | 4 | 4 | 4 | 4 | 2 |

***Table 16. Total number of samples analysed at each time point***

Pro-inflammatory (*IL1A*), chemotactic (*CXCL8, CCL2*) and anti-inflammatory (*IL10*) mRNA transcript levels were significantly higher in the LPS + Hypoxia and LPS groups compared to Hypoxia in the first 6 hours post-insult (p < 0.01) (Figure 22). *IL10* mRNA levels differentiated across all three pathological states; fold-changes were highest in LPS + Hypoxia animals, followed by LPS and Hypoxia at 6 hours (p<0.033). *IL1A* and *IL10* mRNA levels remained elevated for longer in animals receiving LPS + Hypoxia, compared to LPS and Hypoxia groups.

*IL6* mRNA levels were significantly higher in LPS + Hypoxia compared to LPS (p = 0.01) and trend towards increase compared to Hypoxia (p = 0.06) at 3 hours post-insult. *IL6* and *CXCL8* mRNA demonstrated a biphasic response to LPS + Hypoxia, peaking at 3 - 6 hours and at 24 hours post-insult (p < 0.01). Pro-inflammatory gene *TNFA* mRNA decreased in the first 6 hours in all study groups, however increased at 24 hours post-insult in LPS + Hypoxia and LPS groups compared to Hypoxia (p < 0.01). Complete mRNA relative quantification data is listed in Appendix C3.
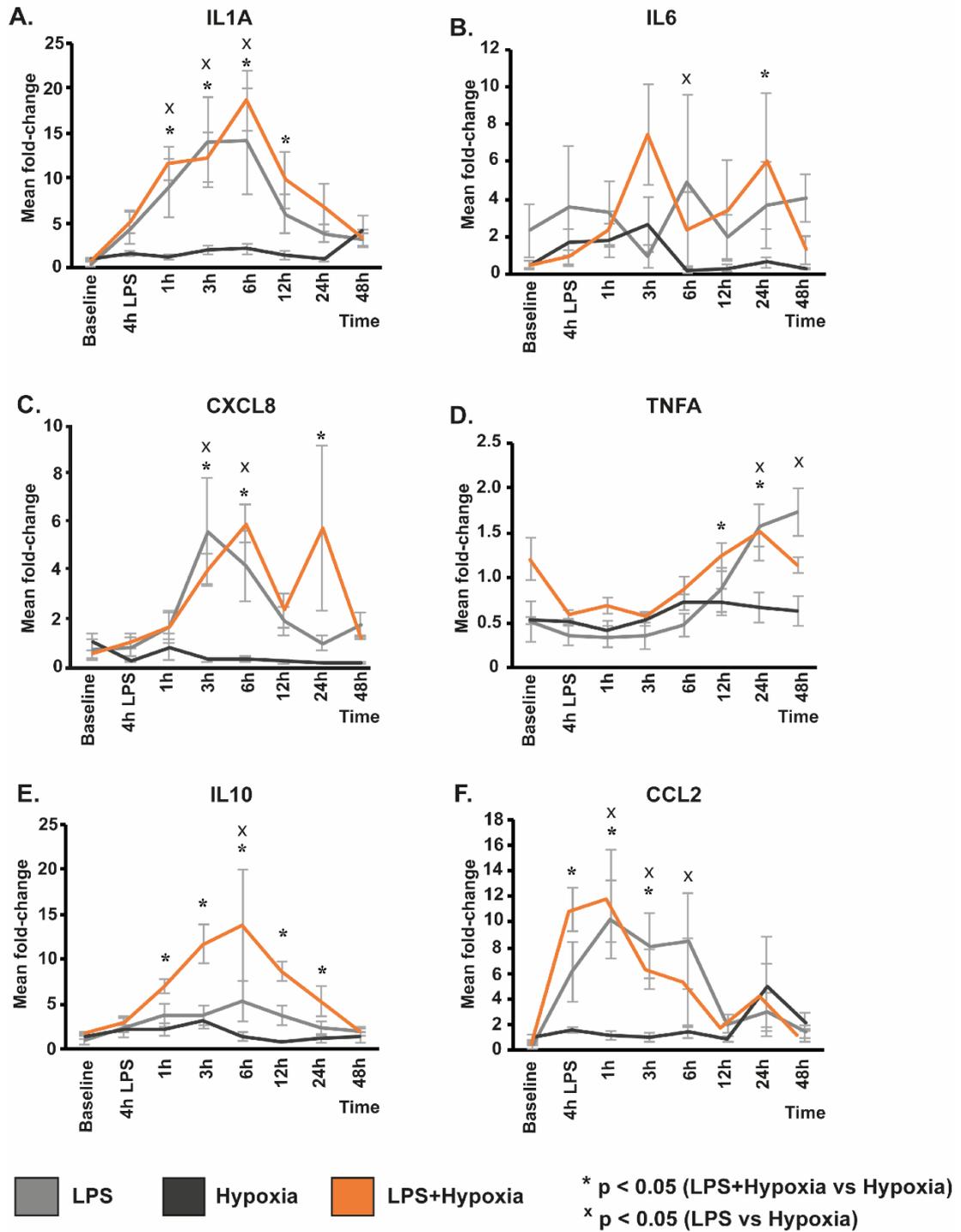
*Figure 22. Serum mRNA transcript levels of pro-inflammatory cytokines (A-D), anti-inflammatory cytokine (E) and chemokine (F) over 48 hours (error bars represent SEM; baseline – 24h, n=15; 48h, n=13).*

*6.2.5 Gene expression for brain-specific biomarkers*

*ENO2* mRNA (neuron-specific enolase) was significantly increased at 1 hour post-insult in LPS + Hypoxia and LPS compared to Hypoxia groups (p = 0.04 and p = 0.01, respectively). At 3 hours, the expression of this transcript was also significantly higher in LPS + Hypoxia animals compared to LPS alone (p = 0.05) (Figure 23).

*MAPT* mRNA (microtubule protein tau) appeared increased in animals receiving LPS at 6 hours and 48 hours (p = 0.04) and LPS + Hypoxia at 24 hours (p < 0.04) compared to Hypoxia alone. Neurotrophic factor *BDNF* mRNA was higher in LPS + Hypoxia compared to Hypoxia animals from 3 hours after insult reaching statistical significance at 24 hours (p = 0.03).

*UCHL1* mRNA (a neuron-specific enzyme) did not distinguish between study groups at any time points. *S100B* mRNA, found in high concentration in glial cells, varied significantly between groups at baseline and remained elevated in animals receiving LPS. *GFAP* mRNA, which encodes the production of a cytoskeletal filament protein and *CRP,* an inflammatory biomarker, were below the threshold for detection in several samples across all study groups and therefore quantification was not possible.
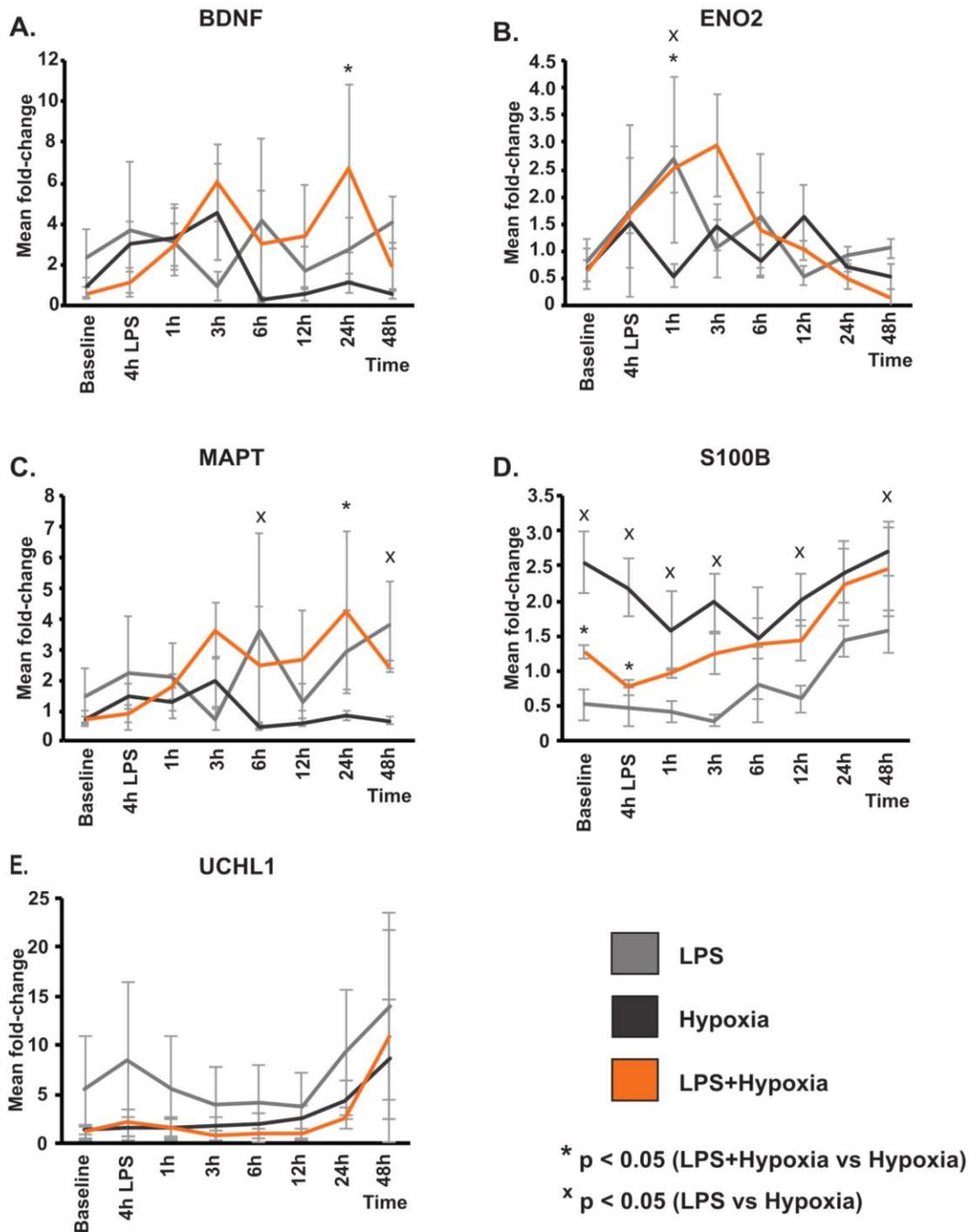
BDNF = Brain derived neurotrophic factor; ENO2 = Enolase 2; MAPT = Microtubule associated protein tau; S100B = s100 protein; UCHL1 = ubiquitin carboxyl-terminal esterase L1; GFAP = Glial Fibrillary Acidic Protein; CRP = C-reactive protein

**Figure 23. Serum mRNA transcript levels of brain-specific proteins over 48 hours (error bars represent SEM; baseline – 24h, n=15; 48h, n=13). Serum GFAP and CRP mRNA was insufficient for analysis.**

*6.2.6 Expression of miRNA*

A total of 4929 miRNAs were analysed and significant changes from baseline were seen in 22 miRNAs (FDR p-value <0.1; see Appendix C4). Overall, significant changes from baseline levels were seen in 12 miRNAs (2 up, 10 down) in the LPS group, 11 miRNAs (all down) in the Hypoxia group and 7 miRNAs (2 up, 5 down) in the LPS + Hypoxia group.

We identified 8 miRNAs that discriminated between study group at specific time points. In the first 6 hours post-hypoxia, hsa-mir-23a-5p and hsa-mir-27a-5p levels were significantly increased in the LPS and LPS + Hypoxia compared to Hypoxia animals at 1, 3 and 6 hours (all $p < 0.01$). Hsa-mir-31-5p and hsa-mir-193-5p levels were significantly lower in LPS + Hypoxia and LPS animals compared to the Hypoxia group immediately post-insult ($p < 0.01$), though this difference was no longer significant by 1 hour ($p = 0.06$) (Figure 24A-D). There were 4 miRNAs that distinguished LPS + Hypoxia from Hypoxia after 24 hours; hsa-mir-181c-3p, hsa-mir-330-3p, hsa-mir-182-5p and hsa-mir-150-5p (Figure 24E-H).
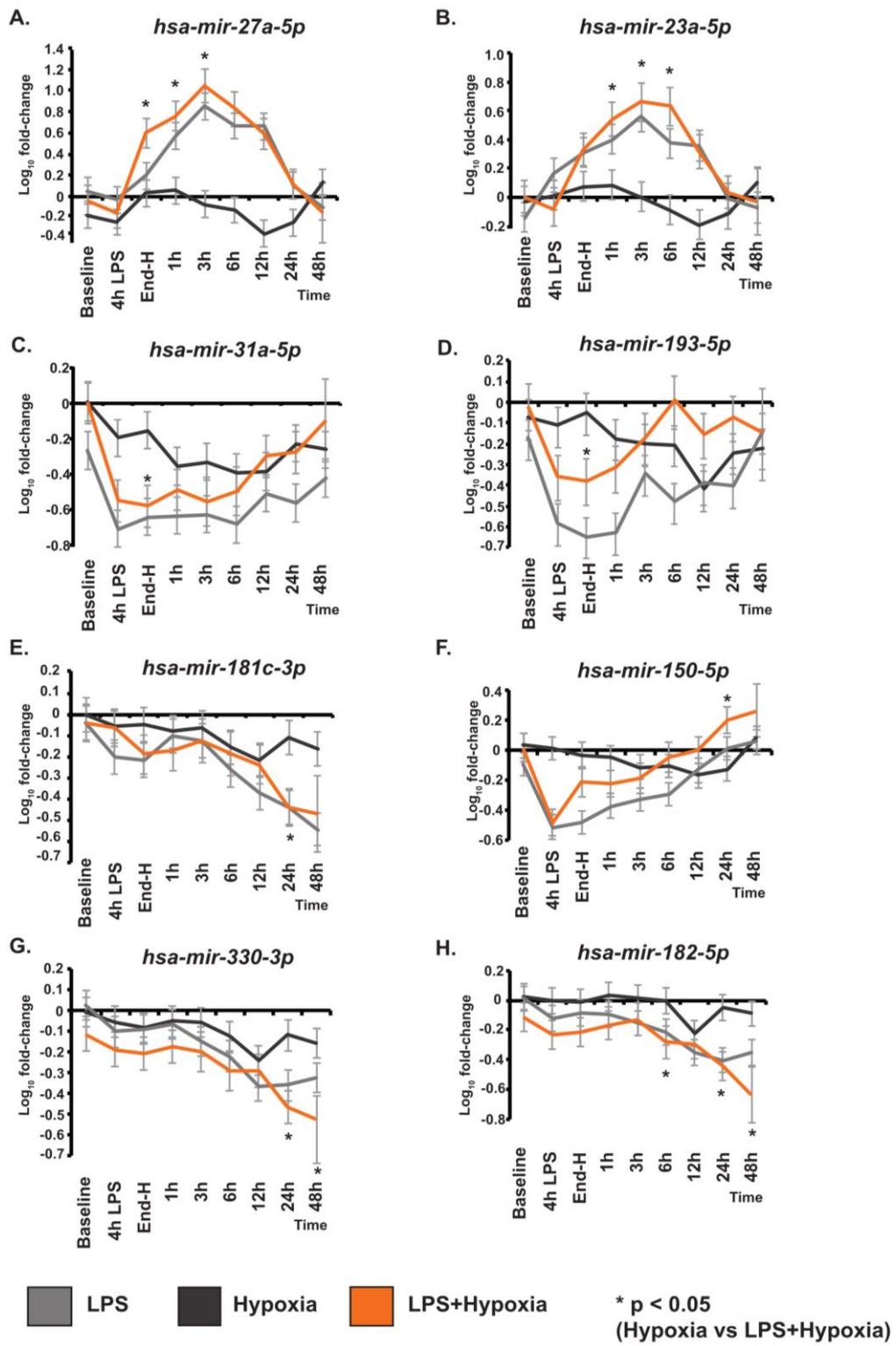
***Figure 24. miRNA that discriminate between Hypoxia and LPS + Hypoxia at early (A-D) and late time points (E-H) (error bars represent SEM; baseline – 24h, n=15; 48h, n=13).***

*6.2.7 Biomarkers of injury severity*

Fold-changes in candidate biomarkers were correlated with overall TUNEL (cell death) to determine whether these mRNA transcripts indicate injury severity. Overall cell death correlated significantly with *TNFA* (R = 0.69, p < 0.01) and *S100B* (R = 0.53, p = 0.04) mRNA at 1 hour post-insult and negatively correlated with *ENO2* mRNA at 48 hours (R = -0.69, p = 0.01). *IL10* mRNA was not associated with overall cell death (Table 17).

Out of the 22 miRNAs that changed significantly from baseline, there were 9 miRNA that significantly correlated with cell death within 6 hours of insult; ssc-mir-31 (R = 0.59, p = 0.027), ssc-mir-199a-5p (R = 0.79, p = 0.001) and hsa-mir-214-5p (R = 0.66, p = 0.01) at 1 hour; hsa-mir-150-5p (R = 0.63, p = 0.02) at 3 hours; ssc-mir-4334-3p (R = 0.79, p = <0.01) and hsa-mir-181c-3p (R = 0.73, p < 0.01) at 6 hours post-insult (Table 18). Notably, hsa-mir-193a-5p and ssc-mir-99b were strongly associated with neuronal cell death at 1, 3 and 6 hours post-insult (R > 0.70, p < 0.01 at 6 hours).

|  | 1h | | 3h | | 6h | | 12h | | 24h | | 48h | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
|  | Correlation | p | Correlation | p | Correlation | p | Correlation | p | Correlation | p | Correlation | p |
| *BDNF* | 0.14 | 0.610 | 0.20 | 0.477 | -0.23 | 0.415 | -0.16 | 0.578 | 0.04 | 0.880 | -0.34 | 0.251 |
| *CCL2* | -0.21 | 0.457 | -0.26 | 0.356 | -0.31 | 0.268 | -0.28 | 0.313 | 0.06 | 0.829 | 0.31 | 0.308 |
| *TNFA* | **0.69** | **0.004*** | **0.53** | **0.043*** | 0.30 | 0.274 | 0.30 | 0.278 | 0.01 | 0.961 | -0.31 | 0.302 |
| *NSE* | -0.18 | 0.528 | 0.20 | 0.482 | -0.15 | 0.596 | 0.09 | 0.746 | -0.12 | 0.658 | **-0.69** | **0.010*** |
| *IL10* | 0.17 | 0.550 | 0.20 | 0.471 | -0.04 | 0.887 | 0.06 | 0.828 | 0.11 | 0.686 | -0.10 | 0.751 |
| *IL1A* | -0.01 | 0.974 | -0.12 | 0.661 | -0.12 | 0.669 | 0.25 | 0.376 | 0.31 | 0.267 | 0.01 | 0.986 |
| *IL6* | -0.10 | 0.734 | 0.20 | 0.480 | -0.24 | 0.384 | -0.20 | 0.481 | -0.06 | 0.827 | -0.39 | 0.194 |
| *IL8* | 0.11 | 0.707 | -0.31 | 0.259 | -0.03 | 0.909 | 0.03 | 0.904 | 0.28 | 0.319 | -0.41 | 0.167 |
| *Tau* | -0.06 | 0.820 | 0.23 | 0.408 | -0.22 | 0.431 | -0.14 | 0.627 | -0.05 | 0.858 | -0.33 | 0.274 |
| *S100B* | **0.53** | **0.043*** | 0.27 | 0.338 | -0.06 | 0.832 | 0.22 | 0.437 | 0.07 | 0.807 | 0.33 | 0.263 |
| *UCHL1* | -0.16 | 0.568 | -0.15 | 0.591 | -0.13 | 0.654 | -0.09 | 0.742 | -0.16 | 0.562 | -0.05 | 0.882 |

*Table 17. Correlation of overall cell death (TUNEL-positive cells) with cytokine and brain-specific mRNA transcript levels at specific time points post-HI*

|  | 1h | | 3h | | 6h | | 12h | | 24h | | 48h | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
|  | Correlation | p | Correlation | p | Correlation | p | Correlation | p | Correlation | p | Correlation | p |
| *hsa-mir-150-5p* | 0.34 | 0.235 | **0.63** | **0.020*** | 0.52 | 0.068 | 0.28 | 0.332 | 0.07 | 0.805 | 0.13 | 0.714 |
| *hsa-mir-27a-5p* | 0.21 | 0.461 | 0.23 | 0.444 | 0.25 | 0.405 | 0.11 | 0.700 | 0.13 | 0.657 | 0.38 | 0.250 |
| *hsa-mir-23a-5p* | 0.11 | 0.706 | 0.12 | 0.686 | 0.55 | 0.052 | 0.18 | 0.545 | 0.30 | 0.300 | 0.28 | 0.400 |
| *hsa-mir-181c-3p* | 0.12 | 0.681 | 0.46 | 0.118 | **0.73** | **0.005*** | 0.44 | 0.111 | 0.28 | 0.336 | 0.57 | 0.067 |
| *hsa-mir-31-5p* | 0.17 | 0.553 | 0.39 | 0.186 | 0.55 | 0.051 | 0.51 | 0.061 | 0.37 | 0.198 | 0.40 | 0.225 |
| *ssc-mir-31* | **0.59** | **0.027*** | 0.43 | 0.144 | 0.35 | 0.245 | 0.50 | 0.069 | 0.13 | 0.659 | 0.22 | 0.514 |
| *hsa-mir-330-3p* | -0.13 | 0.654 | 0.05 | 0.878 | 0.33 | 0.264 | 0.32 | 0.268 | 0.18 | 0.531 | 0.47 | 0.140 |
| *ssc-mir-199a-5p* | **0.79** | **0.001*** | 0.50 | 0.085 | 0.54 | 0.059 | 0.20 | 0.494 | 0.24 | 0.415 | 0.32 | 0.333 |
| *hsa-mir-193a-5p* | **0.60** | **0.023*** | **0.69** | **0.009*** | **0.88** | **<.0001*** | **0.55** | **0.040*** | 0.48 | 0.081 | -0.20 | 0.559 |
| *hsa-mir-339-5p* | **0.48** | **0.080*** | 0.52 | 0.070 | **0.82** | **0.001*** | 0.33 | 0.251 | **0.57** | **0.033*** | 0.46 | 0.154 |
| *hsa-mir-214-3p* | **0.66** | **0.011*** | **0.61** | **0.027*** | **0.74** | **0.004*** | **0.56** | **0.038*** | 0.32 | 0.264 | 0.14 | 0.676 |
| *ssc-mir-99b* | **0.68** | **0.008*** | 0.38 | 0.202 | **0.83** | **0.000*** | 0.17 | 0.572 | 0.17 | 0.569 | **0.74** | **0.009*** |
| *ssc-mir-215* | 0.21 | 0.478 | 0.22 | 0.465 | 0.21 | 0.486 | 0.22 | 0.456 | 0.13 | 0.656 | -0.06 | 0.862 |
| *ssc-mir-339* | 0.44 | 0.117 | 0.44 | 0.129 | **0.81** | **0.001*** | 0.30 | 0.296 | 0.32 | 0.268 | 0.45 | 0.161 |
| *hsa-miR-5100* | -0.31 | 0.275 | -0.27 | 0.369 | -0.28 | 0.348 | -0.20 | 0.493 | -0.12 | 0.671 | -0.30 | 0.375 |
| *ssc-miR-339-5p* | 0.38 | 0.184 | 0.37 | 0.216 | **0.76** | **0.003*** | 0.26 | 0.379 | 0.32 | 0.259 | 0.39 | 0.236 |
| *ssc-miR-4334-3p* | **0.49** | **0.077*** | 0.53 | 0.061 | **0.79** | **0.002*** | 0.28 | 0.331 | 0.44 | 0.116 | 0.51 | 0.111 |
| *hsa-miR-5582-5p* | -0.36 | 0.206 | -0.35 | 0.244 | 0.24 | 0.437 | 0.05 | 0.872 | **-0.64** | **0.013*** | -0.12 | 0.732 |
| *hsa-miR-7977* | -0.02 | 0.944 | 0.02 | 0.954 | -0.31 | 0.302 | 0.09 | 0.748 | -0.06 | 0.826 | -0.30 | 0.374 |
| *hsa-miR-28-3p* | 0.31 | 0.283 | 0.34 | 0.259 | 0.40 | 0.178 | 0.23 | 0.424 | 0.16 | 0.585 | 0.38 | 0.255 |
| *hsa-miR-182-5p* | 0.08 | 0.777 | 0.27 | 0.364 | 0.35 | 0.239 | 0.28 | 0.340 | 0.20 | 0.487 | 0.48 | 0.135 |
| *ssc-miR-181d-5p* | 0.18 | 0.545 | 0.22 | 0.465 | 0.41 | 0.161 | 0.22 | 0.447 | 0.06 | 0.833 | 0.40 | 0.226 |

***Table 18. Correlation of overall cell death (TUNEL-positive) with miRNA at specific time points post-HI (*p<0.05)***

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## 6.3 Discussion

Using a novel pre-clinical model of inflammation-sensitised NE, this study represents the first comprehensive serial analysis of serum cytokine and brain-specific biomarkers to discriminate between hypoxia and inflammation-sensitised hypoxia. Within the critical 6 hour therapeutic window of initiating HT, *IL1A, CXCL8, IL10, CCL2* and *ENO2* mRNA levels increased in LPS + Hypoxia and LPS compared to Hypoxia. *IL10* mRNA was the only biomarker that clearly differentiated between LPS, Hypoxia and LPS + Hypoxia within 6 hours post-insult.

Pro-inflammatory *IL1A, CXCL8, CCL2* and anti-inflammatory *IL10* mRNA were all up-regulated by 3 hours post-hypoxia and clearly discriminated LPS + Hypoxia and LPS from the Hypoxia group. *IL1A* and *IL6* transcript levels peaked at 3 – 6 hours in all study groups, consistent with the timing of tissue expression seen in rodents undergoing HI [242].

A key finding was our observation that the mRNA levels of the anti-inflammatory cytokine *IL10* allowed us to differentiate between all 3 pathological states – *IL10* mRNA levels were highest in LPS + Hypoxia and lowest in the Hypoxia group at 6 hours post-hypoxia. Raised IL10 has previously been identified as a poor prognostic indicator [120], potentially due to immune paralysis [100].

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In our search for an injury-type discriminator, we also focused on brain-specific biomarkers. *ENO2* mRNA was the only brain biomarker that was significantly upregulated in the LPS + Hypoxia group compared to Hypoxia in the first few hours after insult. *ENO2* encodes for NSE, a glycolytic enzyme found in mature central and peripheral neurons. Elevated NSE in the serum [121] and CSF [122] was associated with increased mortality and morbidity in infants with NE. Critically, this biomarker was identified well within 6 hours of injury and therefore provides an opportunity to re-evaluate the need for hypothermia.

Expression patterns of the other brain-specific markers were not as discriminative. We observed that mRNA for *MAPT* (tau) was elevated in LPS + Hypoxia animals compared to Hypoxia at the 24 hour time point only. Similarly, the expression of *BDNF*, a neurotrophin secreted by neurons and astrocytes, was also significantly elevated at 24 hours in the LPS + Hypoxia group. *UCHL1* mRNA, encoding for a neuronal protease, was increased in all study groups however did not differentiate between the different insult types. *S100B* mRNA levels varied significantly at baseline and may reflect changes associated with surgical instrumentation prior to HI. Increased S100B levels have been reported following cardiac surgery as well as generalised (non-head) trauma, including fractures, burns and bruising [243,244]. Although all animals underwent the same surgical procedure, it remains unclear whether these differences in S100B represents variability in animal tolerance to tracheostomy insertion and carotid artery isolation.

Consistent with our findings in the MgSO₄ gene expression study in Chapter 5, transcripts for GFAP were largely undetectable in most samples across all study groups. Serum GFAP protein has previously been identified as a marker of brain injury and is most likely released into the blood stream by dying glial cells. GFAP mRNA however represents the initiation of new protein manufacture and so may vary significantly from its protein counterpart; explaining the absence of GFAP mRNA in our study despite the extensive cell death seen in LPS + Hypoxia animals.

In this study, *TNFA* mRNA was the only cytokine that significantly correlated with overall TUNEL counts, at 1 and 3 hours post-insult and therefore may be an early marker of injury severity. Transcripts for neuron-specific enolase, *ENO2 mRNA,* negatively correlated with cell death at 48 hours post-insult. We have previously observed a significant correlation between serum IL1β, IL10 and CSF TNFα with MRS biomarkers of injury (Lac/NAA) and IL1β and IL8 with TUNEL positive cells in the thalamus in piglets post-HI [103]. Serum mRNA transcripts of these cytokines however did not correlate with overall neuronal cell death.

MiRNAs are essential in the development of the central nervous system [146]. Using microarray analysis, we were able to explore a vast library of known miRNA and identified 4 promising candidates as early differentiating pathological biomarkers of infection sensitised hypoxia: hsa-mir-23a; hsa-mir-27a; hsa-mir-31a-5p; and hsa-mir-193-5p. Two of these, miR-23 and miR-27 together with miR-24 form a cluster that regulate multiple aspects of T cell

activation and differentiation [245]. In this role, miR-27a has previously been shown to promote pro-inflammatory responses in T cells from patients with multiple sclerosis and overexpression in mice was associated with an elevated IFNy response [246]. Analogous to miR-31a-5p, miR-31 has been identified as a regulator of the transcription factors, hypoxia-inducible-factor 1α (HIF1 α) and NF-κB. Both hsa-mir-31 and hsa-mir-193-5p correlated with neuronal cell death 1 hour post-insult and therefore may also be markers of injury severity.

Hsa-mir-181c-3p, hsa-mir-330-3p, hsa-mir-182-p and hsa-mir-150-5p were down-regulated in all study groups, particularly in the LPS and LPS + Hypoxia groups compared to Hypoxia animals at 24 hours post-insult. The differential expression in miRNA levels following LPS alone suggest that these miRNA may be useful in identifying infants with ongoing inflammatory or infective processes. Mir-150-5p is highly expressed in mature B cells, T cells and NK cells; and is thought to play a critical role in immune cell differentiation. Mir-150-5p has also been identified as a prognostic marker following acute ischaemic stroke [247], consistent with the positive correlation with cell death seen in this study. Mir-181c-3p has been linked to the hypoxic response previously [248], downstream of HIF1α [249]. We identified a positive correlation between this miRNA and neuronal cell death, suggesting a potential role in the pathogenesis of neurological injury.

The main strength of this study is the serial investigation of several biomarkers over 48 hours, with particular focus at early time points to help inform decisions on the suitability of therapeutic hypothermia. Gene expression point-of-care

diagnostics is currently in development and this study demonstrates the feasibility and clinical applicability of mRNA-based biomarkers.

The main limitation of the study is the small numbers of animals, particularly at the 48 hour time-point due to high mortality associated with LPS + Hypoxia. The primary focus of the study was on early changes in biomarker expression, particularly within the 6 hour therapeutic window when considering therapeutic hypothermia. As we were able to obtain mRNA and miRNA for 15/16 animals up to 24 hours, it was considered unethical to undertake further experiments.

Animals receiving LPS+Hypoxia were critically unwell with severe haemodynamic compromise and multi-organ failure. This may have confounded the observed cytokine profile in these animals. Given the small group size, it may be challenging to unpick whether the cytokine mRNA levels were driven by neuro-inflammation, systemic injury or a combination of both.

As discussed in Section 5.3, the absence of gene expression data for sham experiments was also a limiting factor. Any trauma initiates an inflammatory response and the impact of tracheostomy and carotid occluder insertion remains uncertain. It is plausible that surgery itself may represent an additional sensitising event that either pre-conditioned or exacerbated the subsequent HI. Exploring this relationship might help refine our piglet studies, providing insight into the most appropriate timing of HI post-surgery in order to minimise its influence.

Finally, timing of inflammation-sensitisation is critical and significantly influences the vulnerability of the brain to hypoxia injury [93,95]. Further study is necessary to establish the impact of timing of LPS-sensitisation on cytokine expression following hypoxia injury.

### 6.4 Conclusion

We have shown that the gene expression of specific cytokines, brain biomarkers and miRNA can be used to differentiate a predominately inflammation-mediated injury from hypoxia within 6 hours of a sentinel event.

Interestingly, the majority of inflammatory cytokine mRNA and brain biomarkers tested increased significantly with LPS exposure, with no clear additive effect when combined with hypoxia. Therefore differentiating infection without hypoxia from infection with hypoxia may prove challenging. This highlights the need to correlate any investigation with clinical history and examination to ensure accurate diagnosis. Although the signs of early onset sepsis may mimic signs of neonatal encephalopathy, there may be other indicators in the perinatal history that support a diagnosis of HIE rather than infection e.g. onset of symptoms from birth, abnormal cord gases or history of sentinel event.

The identification of infants with inflammation-sensitisation prior to perinatal asphyxia provides a unique opportunity to initiate therapies specific to this injury type and potentially improve long-term outcomes beyond hypothermic neuroprotection.

**Chapter 7**



**Discussion**

### 7.1 Preterm vs term and adult magnesium neuroprotection

Magnesium has been extensively investigated in several pathological states and age groups and shown greatest promise in fetal neuroprotection. Following a series of large randomised control trials, antenatal $MgSO_4$ is now well established as a neuroprotective agent to reduce the risk of cerebral palsy in preterm infants.  Its role in term NE however remains controversial. The reasons why magnesium has proven so effective in preterm neuroprotection compared to term NE and adults is likely multifactorial.

Trials of preterm neuroprotection have typically involved the use of a magnesium bolus and infusion, resulting in a rapid rise and maintenance of high serum concentrations. This regimen differs from studies in term infants which have exclusively used a single or repeated bolus dose. Bolus administration results in peaks and troughs in serum magnesium concentration, limiting exposure to suprasystemic magnesium.

Timing is a key factor in neuroprotective interventions. Preterm neuroprotection involves the antenatal administration of magnesium to women in threatened preterm labour. This exposes the unborn fetus to raised serum magnesium prior to the HI associated with preterm birth and resuscitation. The risks of adverse neurological outcomes following preterm delivery are well documented and therefore pre-emptive treatment with $MgSO_4$ is justified. In the case of term NE, time is needed postnatally to diagnose NE as well as establish its severity and need for treatment. Therefore, neuroprotective

interventions in term infants realistically can only be commenced after birth, which may be too late to prevent initiation of the excitotoxic cascade. This study has also highlighted the challenges in achieving raised CSF magnesium levels despite rapidly achieving and maintaining twice normal serum concentrations.

Finally, there are significant maturational changes between the preterm, term and adult brain. The developing brain undergoes rapid development in the third trimester, involving neuronal migration, synaptogenesis, dendrite and axonal growth as well as myelination. Myelination is dependent on the proliferation of immature pre-oligodendrocytes that deposit myelin around axons during maturation. Pre-oligodendrocytes are particularly vulnerable to excitotoxic and oxidative injury and therefore may be more receptive to magnesium NMDA receptor blockade. Furthermore, neuroprotective interventions in the developing brain may result in a more positive long-term benefit due to its developmental plasticity and potential for rewiring.

### 7.2 Role of magnesium neuroprotection in resource-limited settings

Intrapartum-related deaths represent the third leading cause of mortality under-five worldwide and perinatal mortality disproportionately affects countries with fewest resources. Although therapeutic hypothermia has a profound disease-altering effect on brain injury, the requirements of intensive care renders it unfeasible in the majority of low- and middle-income countries. Studies of therapeutic hypothermia in these settings have failed to show

significant benefit, though this could be due to the limited availability of equipment to help stratify severity of HIE (e.g. no aEEG) as well as the higher prevalence of infective co-morbidities and poor staffing that leads to delays in obstetric intervention.

Magnesium sulphate has great potential for use in resource restricted settings due to its ease of storage, widespread availability and low cost. Implementing a bolus and infusion regimen however may be challenging due to the need for infusion pumps, close physiological monitoring and testing of serum concentrations. Magnesium may however have a role in middle-income countries where staffing and resources are sufficient to initiate this therapy safely. Further pre-clinical study establishing the safety and efficacy of magnesium in an inflammation-sensitised model of NE would be highly relevant in these settings where prevalence of perinatal infection is highest. It would be worthwhile investigating the efficacy of a bolus dosing regimen to facilitate translation to hospitals without intensive care settings.

### 7.3 Conclusions

#### 7.3.1 MgSO₄ in combination with moderate hypothermia is not more effective than hypothermia alone (Hypothesis 1 – refuted)

In this study we set out to determine whether raising serum magnesium to suprasystemic levels would provide additional neuroprotection when given in combination with the standard treatment, therapeutic hypothermia. The overall reduction in neuronal cell death at 48 hours and improvement in aEEG scoring in moderate to severe HI suggests magnesium has a biological effect. However, the absence of improvement seen on our primary outcome (MRS Lac/NAA), a robust prognostic marker, suggests that this improvement is incremental and would not translate to a meaningful long-term benefit. In view of this, there is insufficient data to support this hypothesis. Further pre-clinical refinement is needed prior to translation into clinical trials. This may include optimising $MgSO_4$ dosing to achieve higher serum concentrations or exploring alternative delivery mechanisms and / or drug preparations to facilitate increased magnesium CSF penetration.

#### 7.3.2 MgSO₄ does not alter the gene expression of pro-inflammatory cytokines (Hypothesis 2 – refuted)

Inflammation is a key mechanism of injury in the hours and days following perinatal asphyxia and prior sensitisation renders the immature brain particularly vulnerable to HI. We did not observe any significant difference in cytokine or iNOS gene expression when $MgSO_4$ was administered in

combination with hypothermia in animals with moderate to severe NE. Furthermore, microglial activation and astrogliosis were not significantly different between study groups in overall brain histology. Taken together, our data does not support the hypothesis that $MgSO_4$ provides additional anti-inflammatory benefit beyond hypothermia in animals exposed to moderate to severe HI. It is plausible that hypothermia, which itself has immunomodulatory properties, masked the anti-inflammatory effects of $MgSO_4$.

Small animal data suggests that magnesium inhibits NF-kB nuclear translocation and therefore attenuates the subsequent inflammatory cascade. Other NF-kB inhibitors such as Tat-NEMO-binding domain (TAT-TBD) peptides and ammonium pyrrolidinedithiocarbamate (PDTC) have shown potential as neuroprotective agents in pre-clinical studies [250], though no agents have been assessed in large animal or human trials. It remains unclear whether magnesium provides significant NF-kB inhibition, given the small level of neuroprotection observed in this study.

Developing alternative neuroprotective strategies for inflammation-sensitised NE is highly relevant in low income settings where hypothermia is unavailable and / or unsafe, and prevalence of perinatal infection is greatest. Furthermore, hypothermic neuroprotection may have limited efficacy in this subgroup of NE. The next phase in magnesium neuroprotection should include exploring its safety and efficacy in an LPS-sensitised large animal model of NE.

### 7.3.3 Serum biomarkers can identify the infants with NE with prior exposure to infection and inflammation (Hypothesis 3 – confirmed)

Identifying infants with dual inflammatory and hypoxia injury is a significant challenge. Our data supports the hypothesis that a panel of serum cytokine and brain mRNA biomarkers can unpick this pathologically heterogeneous group of infants, differentiating hypoxia, inflammation and inflammation-sensitised hypoxia brain injury. Establishing the prevailing mechanism of injury following perinatal asphyxia in the first 6 hours after birth is essential given that that hypothermic neuroprotection is time-sensitive and may not be the most appropriate treatment modality in a predominately inflammation mediated brain injury. This data provides us with an opportunity to further interrogate pathophysiological mechanisms within the different injury subtypes as well as assess the efficacy of patient-specific interventions.

### 7.3.4 Conclusion

In conclusion, our data demonstrates that raising serum magnesium to twice baseline levels does not provide significant benefit over therapeutic hypothermia. The minor improvement in neuroprotection seen on histology, coupled with EEG recovery in moderate to severe HI, suggest a biological effect which may be optimised in future pre-clinical studies.

Optimisation of magnesium neuroprotection may lie in targeting a higher serum concentration (2.0-2.5 mmol/L), with regular point-care testing to ensure serum levels do not reach toxic concentrations. Furthermore, it would be

important to establish whether a longer duration of hypothermia would enhance or abolish the observed neuronal rescue. Finally, establishing the effects of magnesium exposure on cytokine protein production in different injury sub-types as well as a sham group; and correlating these with MRS and histological markers of injury may provide further insight into whether magnesium is truly anti-inflammatory.

Currently, no single agent has been shown to improve hypothermic neuroprotection in a clinical trial. The key to developing successful adjuncts may lie in refining our diagnostic criteria for infants with NE in order to apply a patient-specific cocktail of agents that work incrementally and synergistically.

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# **Appendices**

## *Appendix A: List of Publications*

Publications associated with this project are listed below. The published manuscripts themselves are included at the end of the thesis.

A1        Melatonin as an adjunct to therapeutic hypothermia in a piglet model of neonatal encephalopathy: A translational study. (Neurobiol Dis., Jan 2019)

         High-Dose Melatonin and Ethanol Excipient Combined with Therapeutic Hypothermia in a Newborn Piglet Asphyxia Model. (Scientific reports, Mar 2020)

A2        Acute LPS sensitization and continuous infusion exacerbates hypoxic brain injury in a piglet model of neonatal encephalopathy. (Scientific reports, Jul 2019)

A3        Using animal models to improve care of neonatal encephalopathy. (Arch Dis Child Educ Pract Ed., Oct 2016)

A4        Magnesium as a Neuroprotective Agent: A Review of Its Use in the Fetus, Term Infant with Neonatal Encephalopathy, and the Adult Stroke Patient. (Dev Neurosci., Feb 2018)

A5        Short-term effects of early initiation of magnesium infusion combined with cooling after hypoxia-ischemia in term piglets. (Pediatr Res., Dec 2019)

A6        Serial blood cytokine and chemokine mRNA and microRNA over 48 h are insult specific in a piglet model of inflammation-sensitized hypoxia-ischaemia. (Pediatr Res., Jun 2020)

### Appendix B: Magnesium study data

*B1. Serum and CSF Magnesium Levels (mmol/L)*

<u>Mg + HT</u>

| Animal: | LWP478 | LWP 480 | LWP 485 | LWP 488 | LWP 474 | LWP 498 | LWP 503 | LWP 511 |
|---|---|---|---|---|---|---|---|---|
| **Serum** | | | | | | | | |
| Baseline | 0.71 | 0.7 | 0.76 | 0.82 | 0.81 | 0.65 | 0.61 | 0.69 |
| End-HI | 0.92 | 0.81 | 0.82 | 0.86 | 1.06 | 0.66 | 0.75 | 0.83 |
| 2h | 1.76 | 1.7 | 1.56 | 1.64 | 1.72 | 1.58 | 1.43 | 1.6 |
| 3h | 1.73 | 1.6 | 1.49 | 1.63 | 1.59 | 1.6 | 1.44 | 1.62 |
| 6h | 1.55 | 1.55 | 1.44 | 1.62 | 1.62 | 1.54 | 1.44 | 1.6 |
| 12h | 1.75 | 1.4 | 1.41 | 1.51 | 1.67 | 1.42 | 1.41 | 1.47 |
| 18h | 1.57 | 1.41 | 1.55 | 1.32 | 1.65 | 1.43 | 1.32 | 1.7 |
| 24h | 1.45 | 1.55 | 1.62 | 1.38 | 1.55 | 1.24 | 1.33 | 1.99 |
| 30h | 1.56 | 1.29 | 1.45 | 1.39 | 1.67 | 1.25 | 1.38 | 1.9 |
| 36h | 1.45 | 1.2 | 1.55 | 1.33 | 1.66 | 1.25 | 1.35 | 1.82 |
| 42h | 1.35 | 1.23 | 1.41 | 1.36 | 1.8 | 1.23 | 1.4 | 1.85 |
| 48h | 1.4 | 1.36 | 1.42 | 1.4 | 1.76 | 1.29 | 1.36 | 1.98 |
| **CSF** | | | | | | | | |
| Baseline | 1.12 | 1.04 | 0.92 | 1.01 | 1.11 | 1.05 | 1.03 | 1.05 |
| End | 1.14 | 1.32 | 1 | 1.28 | 1.31 | 1.15 | 1.21 | 1.33 |

<u>HT</u>

| Animal: | LWP475 | LWP 481 | LWP 486 | LWP 484 | LWP 489 | LWP 479 | LWP 507* |
|---|---|---|---|---|---|---|---|
| **Serum** | | | | | | | |
| Baseline | 0.76 | 0.68 | 0.77 | 0.67 | 0.7 | 0.75 | n/a |
| End-HI | 1.02 | 0.84 | 0.85 | 0.85 | 0.84 | 0.96 | n/a |
| 2h | 0.75 | 0.8 | 0.77 | 0.79 | 0.63 | 0.79 | n/a |
| 3h | 0.84 | 0.86 | 0.7 | 0.77 | 0.66 | 0.85 | n/a |
| 6h | 0.81 | 0.84 | 0.67 | 0.92 | 0.7 | 0.84 | n/a |
| 12h | 0.84 | 0.87 | 0.69 | 0.72 | 0.65 | 0.82 | n/a |
| 18h | 0.86 | 0.73 | 0.75 | 0.71 | 0.72 | 0.83 | n/a |
| 24h | 0.89 | 0.77 | n/a | 0.7 | 0.81 | 0.87 | n/a |
| 30h | 0.96 | 0.86 | n/a | 0.72 | 0.83 | 0.81 | n/a |
| 36h | 0.94 | 0.83 | n/a | 0.77 | 0.78 | 0.84 | n/a |
| 42h | 1.01 | 0.88 | n/a | 0.82 | 0.84 | 0.85 | n/a |
| 48h | 0.95 | 0.77 | n/a | 0.4 | 0.77 | 0.85 | n/a |
| **CSF** | | | | | | | |
| Baseline | n/a | 1.06 | 1.06 | 0.97 | 1 | 0.97 | n/a |
| End | 1.01 | 1.03 | n/a | 1.08 | 1.02 | 1.06 | n/a |

n/a – no data available at these time points; *LWP507 – serum magnesium
not sent in this animal (samples were used for a separate study)

*B2. Mg+HT vs HT: MRS at 48h (all piglets)*

|  | Difference in means HT vs Mg+HT | Standard error of the difference | Lower 95% C.I. for difference | Upper 95% C.I. for difference | p - value |
|---|---|---|---|---|---|
|  |  |  |  |  |  |
| NTP/epp | 0.018 | 0.017 | -0.019 | 0.054 | 0.317 |
| PCr/Pi | 0.434 | 0.289 | -0.176 | 1.043 | 0.152 |
| Thalamus Lac/NAA ($\log_{10}$) | 0.457 | 0.282 | -0.156 | 1.070 | 0.131 |
| White matter Lac/NAA ($\log_{10}$) | 0.484 | 0.314 | -0.181 | 1.150 | 0.142 |

*B3. Mg+HT vs HT: MRS at 48h (excluding mild HI)*

|  | Difference in means HT vs Mg+HT | Standard error of the difference | Lower 95% C.I. for difference | Upper 95% C.I. for difference | p - value |
|---|---|---|---|---|---|
|  |  |  |  |  |  |
| NTP/epp | 0.023 | 0.019 | -0.019 | 0.064 | 0.254 |
| PCr/Pi | 0.493 | 0.285 | -0.113 | 1.099 | 0.103 |
| Thalamus Lac/NAA ($\log_{10}$) | 0.552 | 0.301 | -0.115 | 1.219 | 0.096 |
| White matter Lac/NAA ($\log_{10}$) | 0.557 | 0.320 | -0.129 | 1.243 | 0.103 |

*B4. Mg+HT vs HT: aEEG over 48h (all piglets)*

|  | Difference in mean aEEG HT vs Mg+HT | Standard error of the difference | Lower 95% C.I. for difference | Upper 95% C.I. for difference | p - value |
|---|---|---|---|---|---|
| 0-6hrs | -0.04 | 0.69 | -1.48 | 1.40 | 0.953 |
| 7-12hrs | -0.38 | 0.69 | -1.82 | 1.06 | 0.591 |
| 13-18hrs | -0.07 | 0.69 | -1.50 | 1.37 | 0.925 |
| 19-24hrs | 0.24 | 0.69 | -1.20 | 1.67 | 0.737 |
| 25-30hrs | 0.52 | 0.70 | -0.94 | 1.97 | 0.468 |
| 31-36hrs | 1.23 | 0.70 | -0.22 | 2.69 | 0.093 |
| 37-42hrs | 1.23 | 0.70 | -0.23 | 2.68 | 0.094 |
| 43-48hrs | 1.27 | 0.70 | -0.19 | 2.72 | 0.084 |

*B5. Mg+HT vs HT: aEEG over 48h (excluding mild HI)*

|  | Difference in mean aEEG HT vs Mg+HT | Standard error of the difference | Lower 95% C.I. for difference | Upper 95% C.I. for difference | p - value |
|---|---|---|---|---|---|
| 0-6hrs | 0.02 | 0.47 | -0.93 | 0.97 | 0.966 |
| 7-12hrs | -0.29 | 0.47 | -1.23 | 0.66 | 0.547 |
| 13-18hrs | 0.02 | 0.47 | -0.92 | 0.97 | 0.960 |
| 19-24hrs | 0.34 | 0.47 | -0.61 | 1.29 | 0.475 |
| 25-30hrs | 0.65 | 0.49 | -0.34 | 1.63 | 0.193 |
| 31-36hrs | **1.44** | **0.49** | **0.45** | **2.43** | **0.005*** |
| 37-42hrs | **1.44** | **0.49** | **0.45** | **2.42** | **0.005*** |
| 43-48hrs | **1.48** | **0.49** | **0.50** | **2.47** | **0.004*** |

*B6. Mg+HT vs HT: Regional and Overall TUNEL count (all piglets)*

|  | Difference in mean $\log_{10}$ TUNEL count HT vs Mg+HT | Standard error of the difference | Lower 95% C.I. for difference | Upper 95% C.I. for difference | p - value |
|---|---|---|---|---|---|
| Cingulate cortex | 0.432 | 0.393 | -0.347 | 1.212 | 0.274 |
| Sensorimotor cortex | 0.038 | 0.393 | -0.742 | 0.817 | 0.924 |
| Hippocampus | -0.023 | 0.406 | -0.828 | 0.782 | 0.956 |
| Periventricular white matter | 0.664 | 0.393 | -0.116 | 1.443 | 0.094 |
| Internal capsule | 0.573 | 0.393 | -0.207 | 1.352 | 0.148 |
| Caudate | 0.209 | 0.393 | -0.571 | 0.988 | 0.596 |
| Putamen | 0.426 | 0.393 | -0.353 | 1.206 | 0.281 |
| Thalamus | 0.469 | 0.393 | -0.310 | 1.249 | 0.235 |
|  |  |  |  |  |  |
| **Overall** | 0.349 | 0.140 | 0.072 | 0.625 | **0.014*** |

*B7. Mg+HT vs HT: Regional and Overall TUNEL count (excluding mild HI)*

| | Difference in mean $\log_{10}$ TUNEL count HT vs Mg+HT | Standard error of the difference | Lower 95% C.I. for difference | Upper 95% C.I. for difference | p - value |
|---|---|---|---|---|---|
| Cingulate cortex | 0.378 | 0.434 | -0.485 | 1.240 | 0.386 |
| Sensorimotor cortex | -0.057 | 0.434 | -0.919 | 0.806 | 0.897 |
| Hippocampus | -0.032 | 0.450 | -0.927 | 0.863 | 0.944 |
| Periventricular white matter | 0.597 | 0.434 | -0.266 | 1.459 | 0.173 |
| Internal capsule | 0.727 | 0.434 | -0.135 | 1.589 | 0.097 |
| Caudate | 0.316 | 0.434 | -0.546 | 1.179 | 0.468 |
| Putamen | 0.607 | 0.434 | -0.255 | 1.470 | 0.165 |
| Thalamus | 0.542 | 0.434 | -0.320 | 1.404 | 0.215 |
| | | | | | |
| **Overall** | 0.385 | 0.154 | 0.078 | 0.691 | **0.014*** |

## Appendix C: Gene Expression Study Data

*C1. Target genes and Assay ID for cytokines, chemokines and brain specific proteins*

| Gene | Assay ID (Thermo Fisher Scientific, UK) | Function |
|------|------|----------|
| IL1A | Ss03391336_m1 | Pro-inflammatory cytokine. |
| IL1B | Ss03393804_m1 | Pro-inflammatory cytokine. |
| CXCL8 | Ss03392435_m1 | Pro-inflammatory cytokine. |
| TNFA | Ss03394333_m1 | Pro-inflammatory cytokine. |
| IL6 | Ss03384604_u1 | Anti- and pro-inflammatory cytokine. |
| IL10 | Ss03382372_u1 | Anti-inflammatory cytokine. |
| CCL2 | Ss03394377_m1 | A chemokine that recruits monocytes, T cells and dendritic cells to sites of injury or infection.. |
| BDNF | Ss03822335_s1 | Neurotrophic factor supports neuronal survival and encourages growth and differentiation. |
| GFAP | Ss03373546_m1 | Cytoskeletal intermediate filament protein present in astrocytes. |
| UCHL1 | Ss03381306_u1 | Neuron specific enzyme concentrated in neuronal cell bodies and dendrites. |
| S100B | AI6RPXB | Calcium binding protein and present in high concentrations in glial cells. |
| MAPT | AI5IRQ3 | Gene for Tau protein, a microtubule associated protein present in neurons of the central nervous system. |
| ENO2 | AJWR24G | Cytosolic enzyme found in central and peripheral neurons as well as neuroendocrine cells. |
| iNOS | Ss03374608_u1 | Syntheses nitric oxide, a potent free radical. An important component of innate immunity. |

*C2. Cytokine and brain biomarker mRNA: Mg + HT vs HT*

HT group:

|  | CCL2 | GFAP | IL10 | IL1B | IL6 | INOS | MAPT (TAU) | NSE | S100B | TNFA |
|---|---|---|---|---|---|---|---|---|---|---|
| **Baseline** | 1.0 | 0.6 | 1.4 | 1.1 | 4.9 | 2.6 | 1.9 | 0.9 | 1.1 | 1.0 |
| **2h** | 0.9 | 0.4 | 1.7 | 0.7 | 2.8 | 2.0 | 1.4 | 1.1 | 0.7 | 0.9 |
| **3h** | 2.8 | 1.0 | 3.2 | 1.5 | 8.2 | 5.0 | 2.9 | 3.6 | 1.2 | 2.7 |
| **6h** | 12.7 | 0.8 | 1.9 | 0.9 | 4.8 | 3.1 | 2.0 | 1.4 | 0.8 | 2.4 |
| **12h** | 6.8 | 1.5 | 0.7 | 0.6 | 0.6 | 0.7 | 0.5 | 0.6 | 0.6 | 1.7 |
| **24h** | 2.3 | 0.6 | 0.9 | 0.8 | 2.3 | 1.6 | 1.3 | 1.0 | 1.4 | 2.4 |
| **48h** | 9.6 | 0.6 | 0.4 | 0.4 | 0.1 | 0.8 | 0.5 | 2.8 | 2.6 | 4.9 |

Mg + HT group:

|  | CCL2 | GFAP | IL10 | IL1B | IL6 | INOS | MAPT (TAU) | NSE | S100B | TNFA |
|---|---|---|---|---|---|---|---|---|---|---|
| **Baseline** | 1.6 | 1.9 | 0.9 | 1.3 | 1.8 | 0.8 | 0.8 | 1.2 | 1.1 | 1.4 |
| **2h** | 0.9 | 0.9 | 3.2 | 1.1 | 24.6 | 8.1 | 8.5 | 1.5 | 1.2 | 1.3 |
| **3h** | 2.1 | 1.4 | 2.4 | 1.3 | 12.2 | 3.4 | 3.3 | 2.1 | 0.8 | 1.4 |
| **6h** | 6.4 | n/a | 1.7 | 0.9 | 16.2 | 2.5 | 1.7 | 1.7 | 0.7 | 2.4 |
| **12h** | 5.4 | 0.9 | 0.5 | 1.0 | 1.6 | 0.7 | 0.6 | 1.0 | 0.8 | 1.8 |
| **24h** | 1.9 | 5.5 | 0.7 | 1.1 | 1.6 | 1.2 | 0.9 | 1.5 | 1.0 | 2.3 |
| **48h** | 1.9 | 0.9 | 0.4 | 0.4 | 2.7 | 0.9 | 1.0 | 1.4 | 1.9 | 1.7 |

*C3. Relative mRNA expression of inflammatory markers and brain specific proteins between study groups*

| | | Baseline | 4hLPS | 1h | 3h | 6h | 12h | 24h | 48h |
|---|---|---|---|---|---|---|---|---|---|
| IL1A | *LPS* | 0.45 | 4.40 | **8.92 $^X$** | **13.97 $^X$** | **14.09 $^X$** | 6.00 | 3.85 | 3.12 |
| | *Hypoxia* | 1.05 | 1.56 | 1.20 | 2.09 | 2.13 | 1.48 | 0.97 | 4.16 |
| | *LPS+Hypoxia* | 0.83 | 5.14 | **11.59*** | **12.26*** | **18.61*** | **9.77*** | 6.79 | 3.29 |
| IL6 | *LPS* | 2.35 | 3.63 | 3.27 | 0.93 | **4.94 $^X$** | 2.01 | 3.70 | 4.07 |
| | *Hypoxia* | 0.49 | 1.72 | 1.82 | 2.67 | 0.17 | 0.33 | 0.64 | 0.31 |
| | *LPS+Hypoxia* | 0.51 | 0.92 | 2.39 | **7.44 $^Y$** | 2.39 | 3.41 | **6.03*** | 1.33 |
| CXCL8 | *LPS* | 0.72 | 0.84 | 1.68 | **5.61 $^X$** | **4.15 $^X$** | 1.90 | 0.98 | 1.72 |
| | *Hypoxia* | 1.05 | 0.25 | 0.82 | 0.30 | 0.33 | 0.24 | 0.19 | 0.15 |
| | *LPS+Hypoxia* | 0.54 | 1.05 | 1.70 | **4.02*** | **5.93*** | 2.35 | **5.75* $^Y$** | 1.22 |
| IL10 | *LPS* | 1.11 | 2.45 | 3.74 | 3.78 | **5.35 $^X$** | 3.83 | 2.42 | 1.94 |
| | *Hypoxia* | 1.44 | 2.26 | 2.23 | 3.11 | 1.39 | 0.82 | 1.12 | 1.33 |
| | *LPS+Hypoxia* | 1.73 | 3.06 | **6.97*** | **11.7* $^Y$** | **13.7* $^Y$** | **8.73* $^Y$** | **5.32*** | 2.06 |
| CCL2 | *LPS* | 0.41 | 6.08 | **10.15$^X$** | **8.12 $^X$** | **6.77 $^X$** | 2.06 | 3.03 | 1.47 |
| | *Hypoxia* | 1.04 | 1.57 | 1.17 | 1.02 | 1.41 | 0.89 | 4.92 | 2.16 |
| | *LPS+Hypoxia* | 0.47 | **10.97*** | **12.04*** | **6.36*** | 5.36 | 1.73 | 4.26 | 1.18 |
| TNFA | *LPS* | 0.51 | 0.35 | 0.34 | 0.36 | 0.47 | 0.87 | **1.58 $^X$** | **1.73 $^X$** |
| | *Hypoxia* | 0.52 | 0.52 | 0.42 | 0.53 | 0.73 | 0.73 | 0.67 | 0.63 |
| | *LPS+Hypoxia* | **1.21*$^Y$** | 0.59 | 0.69 | 0.57 | 0.86 | **1.23*** | **1.51*** | **1.14 $^Y$** |
| ENO2 | *LPS* | 0.84 | 1.75 | **2.70 $^X$** | 1.07 | 1.65 | 0.56 | 0.94 | 1.06 |
| | *Hypoxia* | 0.69 | 1.55 | 0.55 | 1.45 | 0.84 | 1.65 | 0.73 | 0.54 |
| | *LPS+Hypoxia* | 0.65 | 1.72 | **2.51*** | **2.95 $^Y$** | 1.39 | 1.04 | 0.51 | 0.16 |
| BDNF | *LPS* | 2.36 | 3.76 | 3.15 | 0.99 | 4.22 | 1.78 | 2.78 | 4.11 |
| | *Hypoxia* | 0.94 | 3.01 | 3.37 | 4.60 | 0.34 | 0.65 | 1.15 | 0.63 |
| | *LPS+Hypoxia* | 0.64 | 1.16 | 2.99 | 6.05 | 3.01 | 3.41 | **6.70*** | 1.89 |
| UCHL1 | *LPS* | 5.66 | 8.43 | 5.65 | 4.09 | 4.11 | 3.87 | 9.28 | 13.96 |
| | *Hypoxia* | 1.49 | 1.72 | 1.68 | 1.85 | 2.06 | 2.54 | 4.49 | 8.65 |
| | *LPS+Hypoxia* | 1.19 | 2.21 | 1.66 | 0.87 | 1.06 | 1.08 | 2.63 | 10.98 |
| MAPT | *LPS* | 1.49 | 2.24 | 2.13 | 0.72 | **3.59 $^X$** | 1.31 | 2.92 | **3.79 $^X$** |
| | *Hypoxia* | 0.73 | 1.45 | 1.26 | 1.96 | 0.48 | 0.60 | 0.83 | 0.68 |
| | *LPS+Hypoxia* | 0.70 | 0.90 | 1.76 | 3.60 | 2.50 | 2.63 | **4.26*** | 2.43 |
| S100B | *LPS* | **0.52 $^X$** | **0.48 $^X$** | **0.43 $^X$** | **0.30 $^X$** | 0.81 | **0.61 $^X$** | 1.43 | **1.57 $^X$** |
| | *Hypoxia* | 2.55 | 2.19 | 1.59 | 1.99 | 1.47 | 2.01 | 2.41 | 2.70 |
| | *LPS+Hypoxia* | **1.28*** | **0.77*** | 0.98 | 1.25 | 1.40 | 1.45 | 2.24 | 2.46 |

\* = LPS+Hypoxia vs Hypoxia (p<0.05); x = LPS vs Hypoxia; **Y** = LPS+Hypoxia vs LPS

*C4. MicroRNA isolated with significant fold changes from baseline over 48h (filtered to human and porcine species; duplications removed)*

1. Hsa-mir-23a-5p
2. Hsa-mir-27a-5p
3. hsa-miR-28-3p
4. Hsa-mir-99b-5p
5. Ssc-mir-31
6. Hsa-mir-31-5p
7. Hsa-mir-150-5p,
8. Hsa-mir-181c-3p
9. hsa-miR-182-5p
10. ssc-miR-181d-5p
11. Hsa-mir-193a-5p
12. Hsa-mir-199a-5p
13. Hsa-mir-214-3p
14. Hsa-mir- 215-5p
15. Hsa-mir-330-3p
16. Ssc-mir-339
17. Hsa-mir-339-5p
18. ssc-miR-4334-3p
19. hsa-miR-5100
20. hsa-miR-5582-5p
21. hsa-miR-7977

hsa = *homo sapiens* species
ssc = *sus scrofa* species